BIOGEN GB PRIORITY APPLICATION

8018701

Opened Dec 30 2002
Decachetee le
Commissioner of Patents
Commissaire des brevets
In presence of the Examiner
en présence de l'examinateur

This is EXHIBIT FIERS-28
to
the Affidavit of Walter C. Fiers
sworn before me
this 19 th day of November, 2001

Commissioner for Oath or Notary Public

THE PATENT OFFICE,
25 SOUTHAMPTON BUILDINGS,
LONDON.

I, the undersigned, being an officer duly authorised in accordance with Section 62(3) of the Patents and Designs Act, 1907, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of documents as originally filed in connection with the Patent application identified therein.

WITNESS my hand this
1st day of APRIL 19 37

PATENTS ACT 1977

6 JUNE 1980

PATENTS FORM NO. 1/77
(Rules 6, 15, 19)

The Comptroller
The Patent Office
25 Southampton Buildings
London WC2A 1AY

1980
18701

80/18701

REQUEST FOR THE GRANT OF A PATENT

6.6.77

THE GRANT OF A PATENT IS REQUESTED BY THE UNDERSIGNED ON THE BASIS OF THE PRESENT APPLICATION

I Applicant's or Agent's Reference *(Please insert if available)* MF/2

II Title of Invention Recombinant DNA molecules and their use in producing structural gene for human fibroblast interferon

III Applicant or Applicants *(See note 2)*

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Nationality

IV Inventor *(See note 2)* (a) ~~The applicant(s) is/are the inventor(s)~~
or
(b) A statement on Patents Form No. 7/77 will be furnished

V Authorisation of Agent *(See note 4)* MARKS, ELLIS & CO.

VI Address for Service *(See note 5)* 90/92 Chancery Lane
London WC2A 1AB

VII Declaration of Priority *(See note 6)*

Country	Filing date	File number

VIII The Application claims an earlier date under Section 8(3), 12(6), 15(4) or 37(4) *(See note 7)*

Earlier application or patent number ..... and filing date .....

GB1S

IX Check List *(To be filled in by applicant or agent)*

A	The application contains the following number of sheet(s)		B	The application as filed is accompanied by:-	
1	Request	1 Sheet(s)	1	Priority document	~
2	Description	21 Sheet(s)	2	Translation of priority document	~
3	Claim(s)	10 Sheet(s)	3	Request for Search	2
4	Drawing(s)	13 informal Sheet(s)	4	Statement of Inventorship and Right to Apply	~
5	Abstract	1 Sheet(s)	5	Separate Authorisation of	to follow

X It is suggested that Figure No ..... of the drawings (if any) should accompany the abstract when published

XI Signature *(See note 8)*

Marburn Miller & Co

NOTES:

1 This form, when completed, should be brought or sent to the Patent Office together with the prescribed fee and two copies of the description of the invention.

2 The name, address and nationality of each applicant are to be stated in the spaces provided at III. Names of natural persons should be indicated in full. Bodies corporate should be designated by their corporate name. If there are more than two applicants the information concerning the third (and further) applicants should be given on a separate sheet.

3 Where the applicant or applicants is/are the sole inventor or the joint inventors, the declaration (a) to that effect at IV should be completed and the alternative statement (b) deleted. If however this is not the case the declaration (a) should be struck out and a statement will then be required to be filed upon Patents Form No.7/77.

4 If the applicant wishes to appoint an agent, his name and address of his place of business shall be indicated in the spaces available at V and VI; such indication will be considered to be an authorisation for the agent to prosecute the application up to grant of a patent and to service any patent so granted.

5 If no authorised agent is appointed an address for service in the United Kingdom to which all documents and notices may be sent must be stated at VI. It is recommended that a telephone number be provided if available.

6 The declaration of priority at VII should state the date of the previous filing and the country in which it was made and indicate the file number, if available.

7 When an application is made by virtue of section 8(3), 12(6), 15(4) or 37(4) the appropriate section should be identified at VIII and the number of the earlier application or any patent granted thereon identified.

8 An agent may sign only when previously authorised. An express authorisation signed by the applicant(s) must be received by the Patent Office before the expiry of 3 months from the filing date.

9 Attention of applicants is drawn to the desirability of avoiding publication of inventions relating to any article, material or device intended or adapted for use in war (Official Secrets Acts, 1911 and 1920). In addition after an application for a patent has been filed in the Patent Office the comptroller will consider whether publication or communication of the invention should be prohibited or restricted under section 22 of the Act and will inform the applicant if such prohibition is necessary.

10 Applicants resident in the United Kingdom are also reminded that, under the provisions of section 23 applications may not be filed abroad without written permission or unless an application has been filed not less than six weeks previously in the United Kingdom for a patent for the same invention and no direction prohibiting publication or communication has been given or any such direction has been revoked.

PATENTS ACT 1977

PATENTS FORM NO. 7/77
(Rule 15)

The Comptroller
The Patent Office
25 Southampton Buildings
London WC2A 1AY

6 JUNE 1980 18701

STATEMENT OF INVENTORSHIP AND OF RIGHT TO THE GRANT OF A PATENT

I Application No. 80.18701

II Title Recombinant DNA molecules and their use in producing structural genes for human fibroblast interferon

III I/We BIOGEN N.V.

the applicant(s) in respect of the above mentioned application for a patent do hereby declare as follows:

i) I/We believe the person(s) whose names and addresses are stated on the reverse side of this form (and supplementary sheet if necessary) is/are the inventor(s) of the invention in respect of which the above mentioned application is made;

ii) The derivation of my/our right to be granted a patent upon the said application is as follows:

By virtue of a consultancy agreement dated 21st January 1980 between the inventor and the applicants

iii) I/We consent to the publication of the details contained herein to each of the inventors named on the reverse side of this form.

IV *Signature* Mewburn Ellis

MEWBURN ELLIS & CO.

PLEASE TURN OVER

C613

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NOTES

1 The name(s) and address(es) of the inventor(s) are to be inserted in the spaces provided alongside.

2 Where more than 3 inventors are to be named, the names of the 4th and any further inventors should be given on the reverse side of an additional blank copy of Patents Form No.7/77 and attached to this form.

RECOMBINANT DNA MOLECULES AND THEIR USE IN PRODUCING STRUCTURAL GENES FOR HUMAN FIBROBLAST INTERFERON

TECHNICAL FIELD OF INVENTION

This invention relates to recombinant DNA molecules and their use in producing human fibroblast interferon-like polypeptides and genes. The recombinant DNA molecules disclosed herein are characterized by DNA sequences that code for polypeptides whose amino acid sequence and composition are substantially consistent with human fibroblast interferon and which have an immunological or biological activity of human fibroblast interferon. As will be appreciated from the disclosure to follow, the recombinant DNA molecules of this invention may be used in the production of polypeptides useful in antiviral and antitumor or anticancer agents.

BACKGROUND ART

Two classes of interferons ("IF") are known to exist. Interferons of Class I are small, acid stable (glyco)-proteins that render cells resistant to viral infection (A. Isaacs and J. Lindenmann, "Virus Interference I. The Interferon", Proc. Royal Soc. Ser. B., 147, pp. 258-67 (1957) and W. E. Stewart, II, The Interferon System, Springer-Verlag (1979) (hereinafter "The Interferon System")). Class II IFs are acid labile. At present, they are poorly characterized. Although to some extent cell specific (The Interferon System, pp. 135-45), IFs are not virus specific. Instead, IFs protect cells against a wide spectrum of viruses.

Two antigenically distinct species of Class I human interferon ("HIF") are known to exhibit IF activity. One IF species, fibroblast interferon ("F IF"), is produced upon appropriate induction in diploid fibroblast cells. Another IF species, leukocyte interferon ("Le IF")

is produced together with minor amounts of F IF upon appropriate induction in human leukocyte and lymphoblastoid cells. Both are heterogeneous in regard to size, presumably because of the carbohydrate moiety. F IF has been extensively purified and characterized (E. Knight, Jr., "Interferon: Purification And Initial Characterization From Human Diploid Cells", Proc. Natl. Acad. Sci. USA, 73, pp. 520-23 (1976)). It is a glycoprotein of about 20,000 molecular weight (M. Wiranowska-Stewart, et al., "Contributions Of Carbohydrate Moieties To The Physical And Biological Properties Of Human Leukocyte, Lymphoblastoid And Fibroblast Interferons", Abst. Ann. Meeting Amer. Soc. Microbiol., p. 246 (1978)). The amino acid composition of authentic human fibroblast interferon has been reported (E. Knight, Jr., et al., "Human Fibroblast Interferon: Amino Acid Analysis And Amino-Terminal Amino Acid Sequence", Science, 207, pp. 525-26 (1980)). Elucidation of the amino acid sequence of authentic human fibroblast interferon is also in progress. To date, the amino acid sequence of the NH_2 terminus of the authentic mature protein has been reported for the first 13 amino acid residues: Met-Ser-Tyr-Asn-Leu-Leu-Gly-Phe-Leu-Gln-Arg-Ser-Ser... (E. Knight, Jr., et al., supra). Two distinct genes, one located on chromosome 2, the other on chromosome 5, have been reported to code for F IF (D. L. Slate and F. H. Ruddle, "Fibroblast Interferon In Man Is Coded By Two Loci On Separate Chromosomes", Cell, 16, pp. 171-80 (1979)). Other studies, however, indicate that the gene for F IF is located on chromosome 9 (A. Medger, et al., "Involvement Of A Gene On Chromosome 9 In Human Fibroblast Interferon Production", Nature, 280, pp. 493-95 (1979)).

Le IF has likewise been purified and characterized. Two components have been described, one of 21000 to 22000 and the other of 15000 to 18000 molecular weight (K. C. Zoon, et al., "Purification And Partial

Characterization Of Human Lymphoblastoid Interferon", Proc. Natl. Acad. Sci. USA, 76, pp. 5601-605 (1979)). A portion of the amino acid sequence of authentic Le IF has also been determined, i.e., 20 amino acids from the amino terminus of the mature protein (K. C. Zoon, et al., "Amino-Terminal Sequence Of The Major Component Of Human Lymphoblastoid Interferon", Science, 207, pp. 527-28 (1980)).

A comparison of the initial amino acid sequence of authentic F IF and Le IF reveals no detectable homology within the first 13 amino acids. The total amino acid compositions of the two species are also distinct. In addition, degradation of the sugar residues of the two species by periodate indicates that the carbohydrate structure of the two IFs is different (N. Wiranowska-Stewart, et al., supra).

The two species of HIF have a number of different properties. For example, anti-human Le IF antibodies are less efficient against F IF and anti-sera to human F IF have no activity against human Le IF (The Interferon System, p. 151). Le IF displays a high degree of activity in cell cultures of bovine, feline or porcine origin whereas F IF is hardly active in those cells but has been reported to be active in rat cells (P. Duc-Goiran, et al., "Studies On Virus-Induced Interferons Produced By The Human Aminotic Membrane And White Blood Cells", Arch. Ges. Virus Forsch., 34, pp. 232-43 (1971)). In addition, the two IFs result from different mRNA species (and therefore from presumably different structural genes) that code for polypeptides of different primary sequence (R. L. Cavalieri, et al., "Synthesis of Human Interferon By Xenopus laevis Oocytes: Two Structural Genes For Interferon In Human Cells", Proc. Natl. Acad. Sci. USA, 74, pp. 3287-91 (1977)).

Although both Le and F IFs occur in a glycosylated form, removal of the carbohydrate moiety (P. J.

Bridgen, et al., "Human Lymphoblastoid Interferon", J. Biol. Chem., 252, pp. 6585-87 (1977)) or synthesis of IF in the presence of inhibitors which preclude glycosylation (W. E. Stewart, II, et al., "Effect of Glycosylation Inhibitors On The Production And Properties Of Human Leukocyte Interferon", Virology, 97, pp. 473-76 (1979); J. Fujisawa, et al., "Nonglycosylated Mouse L Cell Interferon Produced By The Action Of Tunicamycin", J. Biol. Chem., 253, pp. 8679-79 (1978); E. A. Havell, et al., "Altered Molecular Species Of Human Interferon Produced In The Presence Of Inhibitors of Glycosylation", J. Biol. Chem., 252, pp. 4425-27 (1977); The Interferon System, p. 181) yields a smaller form of IF which still retains most or all of its IF activity.

Both F IF and Le IF may, like many human proteins, be polymorphic. Therefore, cells of particular individuals may produce IF species within each of the more general F IF and Le IF classes which are physiologically similar but structurally slightly different than the prototype of the class of which it is a part. Therefore, while the protein structure of the F IF or Le IF may be generally well-defined, particular individuals may produce IFs that are slight variations thereof.

IF is usually not detectable in normal or healthy cells (The Interferon System, pp. 51-57). Instead, the protein is produced as a result of the cell's exposure to an IF inducer. IF inducers are usually viruses but may also be non-viral in character, such as natural or synthetic double-stranded RNA, intracellular microbes, microbial products and various chemical agents. Numerous attempts have been made to take advantage of these non-viral inducers to render human cells resistant to viral infection (S. Baron and F. Dianzani (eds.), Texas Reports On Biology And Medicine, 35 ("Texas Reports"), pp. 528-40 (1977)). These attempts have not

been very successful. Instead, use of exogenous IF itself is now preferred.

As an antiviral agent, HIF has been used to treat the following: respiratory infections (Texas Reports, pp. 486-96); herpes simplex keratitis (Texas Reports, pp. 497-500; R. Sundmacher, "Exogenous Interferon in Eye Diseases", International Virology IV, The Hague, Abstract nr. W2/11, p. 98 (1978)); acute hemorrhagic conjunctivitis (Texas Reports, pp. 501-10); adenovirus keratoconjunctivitis (A. Romano, et al., ISM Memo I-A8131 (October, 1979)); varicella zoster (Texas Reports, pp. 511-15); cytomegalovirus infection (Texas Reports, pp. 523-27); and hepatitis B (Texas Reports, pp. 516-22). See also The Interferon System, pp. 307-19. In these treatments F IF and Le IF may display different dose/response curves. However, large-scale use of IF as an antiviral agent requires larger amounts of HIF than heretofore have been available.

IF has other effects in addition to its antiviral action. For example, it antagonizes the effect of colony stimulating factor, inhibits the growth of hemopoietic colony-forming cells and interferes with the normal differentiation of granulocyte and macrophage precursors (Texas Reports, pp. 343-49). It also inhibits erythroid differentiation in DMSO-treated Friend leukemia cells (Texas Reports, pp. 420-28). Some cell lines may be considerably more sensitive to F IF than to Le IF in these regards (S. Einhorn and H. Strander, "Is Interferon Tissue-Specific? - Effect Of Human Leukocyte And Fibroblast Interferons On The Growth Of Lymphoblastoid And Osteosarcoma Cell Lines", J. Gen. Virol., 35, pp. 573-77 (1977); T. Kuwata, et al., "Comparison Of The Suppression Of Cell And Virus Growth In Transformed Human Cells By Leukocyte And Fibroblast Interferon", J. Gen. Virol., 43, pp. 435-39 (1979)).

IF may also play a role in regulation of the immune response. For example, depending upon the dose and time of application in relation to antigen, IF can be both immunopotentiating and immunosuppressive in vivo and in vitro (Texas Reports, pp. 357-69). In addition, specifically sensitized lymphocytes have been observed to produce IF after contact with antigen. Such antigen-induced IF could therefore be a regulator of the immune response, affecting both circulating antigen levels and expression of cellular immunity (Texas Reports, pp. 370-74). IF is also known to enhance the activity of killer lymphocytes and antibody-dependent cell-mediated cytotoxicity (R. R. Herberman, et al., "Augmentation By Interferon Of Human Natural And Antibody-Dependent Cell-Mediated Cytotoxicity", Nature, 277, pp. 221-23 (1979); P. Beverley and D. Knight, "Killing Comes Naturally", Nature, 278, pp. 119-20 (1979); Texas Reports, pp. 375-80; J. R. Huddlestone, et al., "Induction And Kinetics Of Natural Killer Cells in Humans Following Interferon Therapy", Nature, 282, pp. 417-18 (1979); S. Einhorn, et al., "Interferon And Spontaneous Cytotoxicity In Man. II. Studies In Patients Receiving Exogenous Leukocyte Interferon", Acta Med. Scand., 204, pp. 477-83 (1978)). Both may be directly or indirectly involved in the immunological attack on tumor cells.

Therefore, in addition to its use as an antiviral agent, HIF has potential application in antitumor and anticancer therapy (The Interferon System, pp. 319-21 and 394-99). It is now known that IFs affect the growth of many classes of tumors in many animals (The Interferon System, pp. 292-304). They, like other anti-tumor agents, seem most effective when directed against small tumors. The antitumor effects of animal IF are dependent on dosage and time but have been demonstrated at concentrations below toxic levels. Accordingly, numerous investigations and clinical trials have been and continue

to be conducted into the antitumor and anticancer properties of HIFs. These include treatment of several malignant diseases such as osteosarcoma, acute myeloid leukemia, multiple myeloma and Hodgkin's disease (Texas Reports, pp. 429-35). In addition, F IF has recently been shown to cause local tumor regression when injected into subcutaneous tumoral nodules in melanoma and breast carcinoma-affected patients (T. Nemoto, et al., "Human Interferons And Intralesional Therapy Of Melanoma And Breast Carcinoma", Amer. Assoc. For Cancer Research, Abs nr. 993, p. 246 (1979)). Significantly some cell lines which resist the anticellular effects of Le IF remain sensitive to F IF. This differential effect suggests that F IF may be usefully employed against certain classes of resistant tumor cells which appear under selective pressure in patients treated with high doses of Le IF (T. Kuwata, et al., supra; A. A. Creasy, et al., "The Role of G_0-G_1 Arrest In The Inhibition Of Tumor Cell Growth By Interferon", Abstracts, Conference On Regulatory Functions Of Interferons, N.Y. Acad. Sci., nr. 17 (October 23-26, 1979)). Although the results of these clinical tests are encouraging, the antitumor and anticancer applications of HIF have been severely hampered by lack of an adequate supply of purified HIF.

At the biochemical level IFs induce the formation of at least 3 proteins, a protein kinase (B. Lebleu, et al., "Interferon, Double-Stranded RNA And Protein Phosphorylation", Proc. Natl. Acad. Sci. USA, 73, pp. 3107-11 (1976); A. G. Hovanessian and I. M. Kerr, "The (2'-5') Oligoadenylate (ppp A2'-5A2'-5'A) Synthetase And Protein Kinase(s) From Interferon-Treated Cells", Eur. J. Biochem., 93, pp. 515-26 (1979)), a (2'-5')oligo(A) polymerase (A. G. Hovanessian, et al., "Synthesis Of Low-Molecular Weight Inhibitor Of Protein Synthesis With Enzyme From Interferon-Treated Cells", Nature, 268,

pp. 537-39 (1977); A. G. Bovanessian and I. M. Kerr, Eur. J. Biochem. supra) and a phosphodiesterase (A. Schmidt, et al., "An Interferon-Induced Phosphodiesterase Degrading (2'-5')oligoisoadenylate And The C-C-A Terminus Of tRNA", Proc. Natl. Acad. Sci. USA, 76, pp. 4788-92 (1979)). Both F IF and Le IF appear to trigger similar enzymatic pathways (C. Baglioni, "Interferon-Induced Enzymatic Activities And Their Role In The Antiviral State", Cell, 17, pp. 255-64 (1979)) and both may share a common active core because they both recognize a chromosome 21-coded cell receptor (M. Wiranowska-Stewart, "The Role Of Human Chromosome 21 In Sensitivity To Interferons", J. Gen. Virol., 37, pp. 629-34 (1977)). The appearance of one or more of these enzymes in cells treated with IF should allow a further characterization of proteins with IF-like activity.

Today, F IF is produced by human cell lines grown in tissue culture. It is a low yield, expensive process. One large producer makes only 40-60 x 10^6 units of crude F IF per year (V. G. Edy, et al., "Human Interferon: Large Scale Production In Embryo Fibroblast Cultures", in Human Interferon (W. R. Stinebring and P. J. Chapple, eds.), Plenum Publishing Corp., pp. 55-60 (1978)). On purification by adsorption to controlled pore glass beads, F IF of specific activity of about 10^6 units/mg may be recovered in 50% yield from the crude cell extracts (A. Billiau, et al., "Human Fibroblast Interferon For Clinical Trials: Production, Partial Purification And Characterization", Antimicrobial Agents And Chemotherapy, pp. 49-55 (1979)). Further purification to a specific activity of about 10^9 units/mg is accomplished by zinc chelate affinity chromatography in about 100% yield (A. Billiau, et al., "Production, Purification And Properties Of Human Fibroblast Interferon", Abstracts, Conference On Regulatory Functions Of Interferons, N.Y. Acad. Sci., nr 29 (October 23-26, 1979)). Because the

specific activity of F IF is so high, the amount of F IF required for commercial applications is low. For example, 100 g of pure IF would provide between 3 and 30 million doses.

Recent advances in molecular biology have made it possible to introduce the DNA coding for specific non-bacterial eukaryotic proteins into bacterial cells. In general, with DNA other than that prepared via chemical synthesis, the construction of such recombinant DNA molecules comprises the steps of producing a single-stranded DNA copy (cDNA) of a purified messenger RNA (mRNA) template for the desired protein; converting the cDNA to double-stranded DNA; linking the DNA to an appropriate site in an appropriate cloning vehicle to form a recombinant DNA molecule and transforming an appropriate host with that recombinant DNA molecule. Such transformation may permit the host to produce the desired protein. Several non-bacterial genes and proteins have been obtained in E. coli using recombinant DNA technology. These include, for example, Le IF (S. Nagata, et al., "Synthesis in E. coli Of A Polypeptide With Human Leukocyte Interferon Activity", Nature, 284, pp. 316-20 (1980)). In addition, recombinant DNA technology has been employed to produce a plasmid said to contain a gene sequence coding for F IF (T. Taniguchi, et al., "Construction And Identification Of A Bacterial Plasmid Containing The Human Fibroblast Interferon Gene Sequence", Proc. Japan Acad. Ser. B, 55, pp. 464-69 (1979)).

However, in neither of the foregoing has the actual gene sequence of F IF been described and in neither has that sequence been compared to the initial amino acid sequence or amino acid composition of authentic F IF. The former work is directed only to Le IF, a distinct chemical, biological and immunological Class I interferon from F IF (cf. supra). The latter report is

based solely on hybridization data. These data do not enable one to determine if the selected clone contains the complete or actual gene sequence coding for F IF or if the cloned gene sequence will be able to express F IF in bacteria. Hybridization only establishes that a particular DNA insert is to some extent homologous with and complementary to a mRNA component of the poly(A)RNA that induces interferon activity when injected into oocytes. Moreover, the extent of any homology is dependent on the hybridization conditions chosen for the screening process. Therefore, hybridization to a mRNA component of poly(A) RNA alone does not demonstrate that the selected DNA sequence is a sequence which codes for F IF or a polypeptide which displays the immunological or biological activity of F IF.

At a seminar in Zurich on February 25, 1980, Taniguchi stated that he had determined the nucleotide sequence for his hybridizing clone. He also stated that the first 13 amino acids coded for by that sequence were identical to that determined by Knight, et al., supra, for authentic F IF. Taniguchi did not disclose the full nucleotide sequence for his clone or compare its amino acid composition with that determined for authentic F IF. Taniguichi has since reported the full nucleotide sequence for his hybridizing clone (T. Taniguichi et al., Gene, 10, pp. 11-15 (1980). The sequence differs by one nucloetide from that described and claimed in British patent application 8011306, filed April 3, 1980, an application to which the present application claims priority. The amino acid sequence reported by Taniguichi is identical to that described and claimed in the foregoing application. Taniguichi has not reported the expression in a bacterial host of polypeptides which display an immunological or biological activity of F IF. It is this expression in a bacterial host of polypeptide(s), which display an immunological or biological activity of

F IF and the methods, polypeptides, genes and recombinant DNA molecules thereof, which characterize this invention.

Nor is this invention addressed as is the apparent suggestion of Research Disclosure No. 18309, pp. 361-62 (1979) to prepare pure or substantially pure IF mRNA before attempting to clone the HIF gene or to produce fibroblast interferon-like polypeptides in bacterial hosts.

DISCLOSURE OF THE INVENTION

The present invention solves the problems referred to by providing at least one recombinant DNA molecule characterized by a structural gene coding for a polypeptide displaying an immunological or biological activity of human fibroblast interferon.

By virtue of this invention, it is possible to obtain polypeptide(s) displaying an immunological or biological activity of F IF for use as antiviral, antitumor or anticancer agents. This invention allows the production of these polypeptides in amounts and by methods hitherto not available.

As will be appreciated from the disclosure to follow, the recombinant DNA molecules of the invention are capable of directing the production, in an appropriate host, of polypeptide(s) displaying an immunological or biological activity of F IF. Replication of these recombinant DNA molecules in an appropriate host also permits the production in large quantities of genes coding for these polypeptides. The molecular structure and properties of these polypeptides and genes may be readily determined. The polypeptides and genes are useful, either as produced in the host or after appropriate derivatization or modification, in compositions and methods for detecting and improving the production of these products themselves and for use in antiviral and antitumor or anticancer agents.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic outline of one embodiment of a process of this invention for preparing a mixture of recombinant DNA molecules, some of which are characterized by inserted DNA sequences that characterize this invention.

Figure 2 is a schematic outline of the initial clone screening process of this invention.

Figure 3 is a schematic outline of one embodiment of a clone screening process using DNA sequences prepared in accordance with the invention.

Figure 4 displays the composite nucleotide sequence of the coding strand of F IF DNA or gene. The sequence is numbered from the beginning of the insert well into the untranslated area of the insert. Nucleotides 65-127 represent a signal sequence and nucleotides 128-625 represent the "mature" fibroblast interferon. The amino acid sequences of the signal polypeptide are depicted above their respective nucleotide sequences; the amino acids of the signal polypeptide being numbered from -21 to -1 and its other mature interferon from 1 to 166. Review of the restriction and fragment analysis data of the F IF DNA present in the deposited clones has resulted in two nucleotides being changed in Figure 4 from Figure 4 of British patent application 8011306, filed April 3, 1980. These are in the untranslated sequence preceding the proposed signal sequence of F IF DNA. These changes do not effect the sequence of F IF DNA on the amino acid sequence of its translation product and do not alter the genes use as an hydridization probe to screen clones for F IF related DNA inserts.

Figure 5 displays the orientation and restriction maps of several plasmids in accordance with this invention.

Figure 6 is a comparison of the amino acid composition of human fibroblast interferon as determined in accordance with this invention and that determined from authentic fibroblast interferon.

Figure 7 displays a restriction map of the F IF gene of this inveption and the sequencing strategy used in sequencing pHFIF3, pHFIF6, and pHFIF7.

Figure 8 is a schematic outline of the construction of recombinant DNA molecule pPLa-HFIF-67-1 of this invention.

Figure 9 is a schematic outline of the construction of recombinant DNA molecule pPLa-HFIF-67-12 and pPLa-HFIF-67-12Δ19 of this invention.

Figure 10 is a schematic outline of the construction of recombinant DNA molecule pPLc-HFIF-67-8 of this invention.

Figure 11 is a schematic outline of the orientation and partial restriction map of pPLa-HFIF-67-12 of this invention.

Figure 12 is a schematic outline of the orientation and partial restriction map of pPLa-HFIF-67-12Δ19 of this invention.

Figure 13 is a schematic outline of the orientation and partial restriction map of pPLc-HFIF-67-8 of this incention.

BEST MODE OF CARRYING OUT THE INVENTION

In order that the invention herein described may be more fully understood, the following detailed description is set forth.

In the description the following terms are employed:

Nucleotide--A monomeric unit of DNA or RNA consisting of a sugar moiety (pentose), a phosphate, and a nitrogenous heterocyclic base. The base is linked to the sugar moiety via the glycosidic carbon (1' carbon of

the pentose) and that combination of base and sugar is called a nucleoside. The base characterizes the nucleotide. The four DNA bases are adenine ("A"), guanine ("G"), cytosine ("C"), and thymine ("T"). The four bases are A, G, C and uracil ("U").

DNA Sequence--A linear array of nucleotides connected one to the other by phosphodiester bonds between the 3' and 5' carbons of adjacent pentoses.

Codon--A DNA sequence of three nucleotides (a triplet) which encodes through mRNA an amino acid, a translation start signal or a translation termination signal. For example, the nucleotide triplets TTA, TTG, CTT, CTC, CTA and CTG encode for the amino acid leucine ("Leu"), TAG, TAA and TGA are translation stop signals and ATG is a translation start signal.

Reading Frame--The grouping of codons during translation of mRNA into amino acid sequences. During translation the proper reading frame must be maintained. For example, the sequence GCTGGTTCTAAG may be translated in three reading frames or phases, each of which affords a different amino acid sequence:

GCT GGT TCT AAG--Ala-Gly-Cys-Lys
G CTG GTT CTA AG--Leu-Val-Val
GC TGG TTC TAA G--Trp-Leu-(STOP)

Polypeptide--A linear array of amino acids connected one to the other by peptide bonds between the α-amino and carboxy groups of adjacent amino acids.

Genome--The entire DNA of a cell or a virus. It includes inter alia the structural genes coding for the polypeptides of the substance, as well as operator, promoter and ribosome binding and interaction sequences, including sequences such as the Shine-Dalgarno sequences.

Structural Gene--A DNA sequence which encodes through its template or messenger RNA ("mRNA") a sequence of amino acids characteristic of a specific polypeptide.

Transcription--The process of producing mRNA from a structural gene.

Translation--The process of producing a polypeptide from mRNA.

Expression--The process undergone by a structural gene to produce a polypeptide. It is a combination of transcription and translation.

Plasmid--A nonchromosomal double-stranded DNA sequence comprising an intact "replicon" such that the plasmid is replicated in a host cell. When the plasmid is placed within a unicellular organism, the characteristics of that organism may be changed or transformed as a result of the DNA of the plasmid. For example, a plasmid carrying the gene for tetracycline resistance (Tet^R) transforms a cell previously sensitive to tetracycline into one which is resistant to it. A cell transformed by a plasmid is called a "transformant".

Phage or Bacteriophage--Bacterial virus many of which consist of DNA sequences encapsidated in a protein envelope or coat ("capsid").

Cloning Vehicle--A plasmid, phage DNA or other DNA sequence which is able to replicate in a host cell, characterized by one or a small number of endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion without attendant loss of an essential biological function of the DNA, e.g., replication, production of coat proteins or loss of promoter or binding sites, and which contain a marker suitable for use in the identification of transformed cells, e.g., tetracycline resistance or ampicillin resistance. A cloning vehicle is often called a vector.

Cloning--The process of obtaining a population of organisms or DNA sequences derived from one such organism or sequence by asexual reproduction.

Recombinant DNA Molecule or Hybrid DNA--A molecule consisting of segments of DNA from different

genomes which have been joined end-to-end outside of living cells and have the capacity to infect some host cell and be maintained therein.

Expression Control Sequence--A sequence of nucleotides that controls and regulates expression of structural genes when operatively linked to those genes.

Referring now to Figure 1, we have shown therein a schematic outline of one embodiment of a process for preparing a mixture of recombinant DNA molecules, some of which include inserted DNA sequences that characterize this invention.

PREPARATION OF POLY(A)RNA CONTAINING HUMAN FIBROBLAST INTERFERON mRNA (F IF mRNA)

The RNA used in this invention was extracted from human VGS cells, a diploid fibroblast cell line which can be propagated in monolayer cultures at 37°C. F IF is produced in these cells on induction with poly(I,C) in the presence of cycloheximide.

For a typical RNA isolation, each of 20 roller bottles of diploid VGS cells in confluent monolayer was "primed" overnight with 100 units/ml F IF and the cultures induced for 1 h with 100 µg/ml poly(I,C) and 50 µg/ml cycloheximide, incubated with cycloheximide (50 µg/ml) for 4 h, harvested by scraping into phosphate-buffered saline and spun down. The cells were lysed for 15 min at 0°C to remove the intact nuclei containing the DNA and to isolate the cytoplasmic RNA by suspending them in hypotonic buffer (10 mM Tris-HCl (pH 7.4), 10 mM NaCl and 1.5 mM $MgCl_2$) and adding NP40 to 1%. Nuclei were removed by pelleting in a Sorvall SS-34 rotor for 5 min at 3000 rpm. Sodium dodecyl sulphate ("SDS") and EDTA were added to the supernatant to 1% and 10 mM, respectively, and the mixture extracted 5 times with 2x vol of 1:1 redistilled phenol and chloroform-isoamyl alcohol (25:1), the aqueous phases containing the RNA being

separated by centrifugation in a Sorvall SS-34 rotor at 8000 rpm for 10 min after each extraction. The RNA was precipitated from the aqueous phase by addition of 1/10 vol of 2 M sodium acetate (pH 5.1) and 2.5 vol ethanol. Usually, 60 to 90 µg of total cytoplasmic RNA was obtained per roller bottle.

Other procedures to extract the cytoplasmic RNA have also been used. For example, the cells were totally lysed after homogenization in 0.2 M Tris-HCl (pH 9.0), 50 mM NaCl, 20 mM EDTA and 0.5% SDS and extracted with phenol-chloroform as above (F. H. Reynolds, et al., "Interferon Activity Produced By Translation Of Human Interferon Messenger RNA In Cell-Free Ribosomal Systems And In Xenopus Oocytes", Proc. Natl. Acad. Sci. USA, 72, pp. 4881-87 (1975)) or the washed cells were suspended in 400 µl 0.1 M NaCl, 0.01 M Tris-HCl (pH 7.5), and 0.001 M EDTA ("NTE buffer") and 2.5 ml 4 M guanidinium-isothiocyanate and 1 M β-mercaptoethanol in 20 mM sodium acetate (pH 5.0) were added and the cells homogenized. The lysate was layered on a 1.3-ml 5.7 M CsCl cushion in a Beckman SW-60 Ti nitrocellulose tube, spun for 17 h at 39000 rpm to pellet the RNA and separate it from DNA, proteins and lipids and the RNA extracted once with phenol-chloroform (J. Morser, et al., "Characterization Of Interferon Messenger RNA From Human Lymphoblastoid Cells", J. Gen. Virol., 44, pp. 231-34 (1979)).

The total RNA was assayed for the presence of F IF mRNA by injection into the cytoplasm of Xenopus laevis oocytes and determination of the F IF activity induced therein (Reynold, et al., supra). The assay was conducted by dissolving the RNA in water and injecting about 50 µl into each oocyte. The oocytes were incubated overnight at room temperture in Barth medium (J. Gurdon, J. Embryol. Exper. Morphol., 20, pp. 401-14 (1968)), homogenized in part of the medium, the debris removed by centrifugation, and the F IF activity of the supernatant

determined. Detection of F IF activity was by reduction of virus-induced cytopathic effect (W. E. Stewart and S. E. Sulkin, "Interferon Production In Hamsters Experimentally Infected With Rabies Virus", Proc. Soc. Exp. Biol. Med., 123, pp. 650-53 (1965)). The challenge virus was vesicular stomatitis virus (Indiana strain) and the cells were human diploid fibroblasts trisomic for chromosome 21 to afford higher F IF sensitivity. F IF activity is expressed relative to the IF reference standard 69/19.

Poly(A) RNA containing F IF mRNA was isolated from the cytoplasmic RNA by adsorption to oligo(dT)-cellulose (type 7; P-L Biochemicals) in 0.4 M NaCl, 10 mM Tris-HCl (pH 7.8), 10 mM EDTA and 0.2% SDS for 10 min at room temperature. RNA aggregation was minimized by heating the RNA for 2 min at 70°C prior to adsorption. After washing the cellulose with the above-mentioned buffer, the poly(A)RNA fraction was eluted with 10 mM Tris-HCl (pH 7.8), 1 mM EDTA and 0.2% SDS. It usually comprised 4-5% of the total RNA, as measured by optical density at 260 nm.

A further purification to enrich the poly(A)RNA in F IF mRNA was effected by formamide-sucrose gradients (T. Pawson, et al., "The Size of Rous Sarcoma Virus mRNAs Active In Cell-Free Translation", Nature, 268, pp. 416-20 (1977)). These gradients gave much higher resolution than non-denaturing sucrose gradients. Usually about 80 µg poly(A) RNA was dissolved in 50% formamide, 100 mM LiCl, 5 mM EDTA, 0.2% SDS and 10 mM Tris-HCl (pH 7.4), heated at 37°C for 2 min to prevent aggregation and loaded on a 5-20% sucrose gradient in a Beckman SW-60 Ti polyallomer tube. After centrifugation at 20°C for 4 1/2 h at 60000 rpm in the Beckman SW-60 Ti rotor with total ^{14}C-labeled eukaryotic RNA serving as size markers, the gradient was fractionated and the optical density of the fractions determined. All RNA

fractions were precipitated twice with 0.5 M NaCl and 2.5 vol ethanol and assayed for interferon mRNA activity as described above. These purification processes result in about a 40-fold enrichment in the F IF mRNA content of the poly(a) RNA.

Alternatively, the oligo(dT)-adsorbed mRNA (60 µg) was fractionated by electrophoresis in a 4% polyacrylamide gel in 7 M urea, 0.1% SDS, 50 mM Tris-borate (pH 8.3), and 1 mM EDTA, the mRNA being dissolved in this buffer and heated 1 min at 55°C before application to the gel. After electrophoresis, sections of 2 mm width were cut from the gel and the RNA eluted from each homogenized gel section, further freed from impurities by adsorption to oligo(dT)-cellulose and assayed for F IF mRNA as before.

Neither the formamide-sucrose gradients nor the polyacrylamide gel fractionation yields pure F IF mRNA. On the contrary, the fractions displaying F IF mRNA activity actually contain a large number of other unrelated mRNA's, which will behave similarly to F IF mRNA throughout the subsequent processes of this invention. Therefore, a very large number of clones which do not contain the DNA information for F IF will be produced and a complex screening procedure will be required to select the one or few F IF related clones from the total population (Figure 1).

SYNTHESIS OF DOUBLE-STRANDED cDNA CONTAINING F IF cDNA

Poly(A) RNA enriched in F IF mRNA was used as a template to prepare complementary DNA ("cDNA"), essentially as described by R. Devos, et al., "Construction And Characterization Of A Plasmid Containing A Nearly Full-Size DNA Copy Of Bacteriophage MS2 RNA", J. Mol. Biol., 129, pp. 595-619 (1979) for the construction of a plasmid containing a DNA copy of bacteriophage MS2 RNA.

Single-stranded cDNA was prepared from the poly(A) RNA by RNA-dependant DNA polymerase (25 units) from avian myeloblastosis virus ("AMV") reverse transcriptase (a gift from Dr. J. Beard, Life Sciences, Gulfport, Florida), initiated by a $(dT)_{10}$ primer (6 µg, Miles) hybridized to the poly(A) tail of the RNA, in 50 µl 50 mM Tris-HCl (pH 8.3), 10 mM $MgCl_2$, 30 mM β-mercaptoethanol, 4 mM $Na_4P_2O_7$, 2.5 µg/µl inactivated bovine serum albumin, dTTP, dATP, dCTP and dGTP, each at 0.5 mM and α-^{32}P-dATP (20 µCi, Amersham). After 30 min at 41°C, the reaction was terminated by the addition of EDTA to 10 mM, the reaction mixture extracted with equal vol of phenol:chloroform:isoamyl alcohol (25:24:1) and the aqueous phase layered on a Sephadex G50 column and eluted in TE buffer (10 mM Tris-HCl (pH 7.5) 1 mM EDTA). The void fractions displaying radioactivity were precipitated by the addition of 10 µg *E. coli* transfer RNA, potassium acetate (pH 5.1) to 0.2 M and 2.5 vol ethanol.

The cDNA population synthesized above is in fact a complex mixture of cDNAs originating from the different mRNAs which were present in the enriched poly(A) mRNA (Figure 1). In addition, because of premature termination by AMV reverse transcriptase, many of the cDNAs are incomplete copies of the various mRNAs in the poly(A) RNA (not shown in Figure 1).

Before rendering the cDNA double-stranded, it is removed from its association to the complementary template RNA by precipitation with ethanol and incubation in TE buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA) with ribonuclease T_1 (10 units, Sankyo Co., Ltd) and pancreatic ribonuclease A (10 µg, Sigma) to 10 µl for 30 min at 37°C (the ribonucleases being free of single-strand-specific endo- and exo-deoxyribonucleases). The removal of the template strand by ribonuclease instead of with alkali avoids possible cDNA mutation by alkali-catalyzed deamination.

The cDNA strand may be rendered double-stranded by DNA polymerase I (A. Efstratiadis, et al., "Enzymatic In Vitro Synthesis Of Globin Genes", Cell, 7, pp. 279-88 (1976)). The 10 µl ribonuclease/cDNA mixture from above was diluted to 20 µl with $MgCl_2$ to 10 mM, DTT to 10 mM, potassium phosphate (pH 6.9) to 100 mM, dATP, dCTP, dTTP, and dGTP each to 0.3 mM, α-^{32}P-dATP (20 µCi, Amersham) and DNA polymerase I (40 units, Biolabs). After 6 h at 15°C, EDTA to 10 mM and SDS to 0.1% were added and the double stranded cDNA isolated by extraction (phenol:chloroform:isoamyl alcohol), chromatography (Sephadex G50) and precipitation of void fractions as before.

To open the single-stranded hairpin loop which remains on the double stranded cDNA structure, the precipitated cDNA was dissolved in 100 µl 0.3 M NaCl, 50 mM sodium acetate (pH 4.5), 10 mM zinc acetate and 2 µg heat-denatured calf thymus DNA and reacted with S1 nuclease (5 units, Sigma) for 30 min at 37°C. Addition of EDTA to 10 mM, extraction with phenol:chloroform:isoamyl alcohol and precipitation of the aqueous phase by the addition of 10 µg E. coli transfer RNA as carrier, 0.2 M sodium acetate (pH 5.1) and 2.5 vol ethanol yielded a blunt-ended double stranded cDNA mixture. This mixture is heterogeneous both as a consequence of the heterogeneity of the poly(A)RNA used as a template to prepare it (Figure 1) and of the premature termination of the cDNA transcripts by the AMV reverse transcriptase (not shown in Figure 1).

To lessen the effect of the latter heterogeneity, the double stranded cDNA was sized by electrophoresis on a 4% polyacrylamide gel in 50 mM Tris-borate buffer (pH 8.3) and 1 mM EDTA, 5'-^{32}P-labelled restriction fragments (ϕX174 (RF)-DNA) serving as size markers. DNA bands of appropriate size (e.g., size classes 800-900 bp, 700-800 bp, 650-700 bp and 550-650 bp) were

selected. Because the double-stranded cDNA prepared from the polyacrylamide gel electrophoresed poly(A) RNA displayed a prominent band about 850 bp, this band was considered to represent the full-length DNA. The bands were eluted by crushing the gel in 0.5 M ammonium acetate and 0.1% SDS and stirring overnight. After the debris had been removed by centrifugation, the DNA was adsorbed to hydroxylapatite powder, loaded on a Sephadex G50 column in 5 mM sodium phosphate (pH 7.5), washed extensively with buffer, eluted with 0.45 M sodium phosphate (pH 7.5) and immediately desalted by the sieving effect of the Sephadex G50 matrix. The fractions containing the eluted DNA, as monitored by the ^{32}P-radioactivity, were precipitated by the addition of 10 µg E. coli transfer RNA, sodium acetate to 0.2 M and 2.5 vol ethanol.

The efficiency of the cDNA preparation described above, is exemplified by a typical experiment where about 2 µg of poly(A) RNA after formamide-sucrose gradient yielded about 16 ng double-stranded cDNA having a size range of 800 to 900 bp.

Again, it must be recognized that this double-stranded cDNA is a mixture of a large number of cDNAs, only a very few of which are F IF cDNA (Figure 1).

CLONING OF DOUBLE-STRANDED DNA

A wide variety of host/cloning vehicle combinations may be employed in cloning the double-stranded cDNA prepared in accordance with this invention. For example, useful cloning vehicles may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences, such as various known derivatives of SV40 and known bacterial plasmids, e.g., plasmids from E. coli including col E1, pCR1, pBR321, pMB9 and their derivatives, wider host range plasmids, e.g., RP4, phage DNAs, e.g., the numerous derivatives of phage λ, e.g., NM 989, and other DNA phages, e.g., M13 and Filamentous single stranded

DNA phages and vectors derived from combinations of plasmids and phage DNAs such as plasmids which have been modified to employ phage DNA or other expression control sequences or yeast plasmids such as the 2 μ plasmid or derivatives thereof. Useful hosts may include bacterial hosts such as E. coli HB 101, E. coli X1776, E. coli X2282, E. coli MRCI and strains of Pseudomonas, Bacillus subtilis, Bacillus stearothermophilus and other bacilli, yeasts and other fungi, animal or plant hosts such as animal (including human) or plant cells in culture or other hosts. Of course, not all host/vector combinations may be equally efficient. The particular selection of host/cloning vehicle combination may be made by those of skill in the art after due consideration of the principles set forth without departing from the scope of this invention.

Furthermore, within each specific cloning vehicle, various sites may be selected for insertion of the double-stranded DNA. These sites are usually designated by the restriction endonuclease which cuts them. For example, in pBR322 the PstI site is located in the gene for β-lactamase, between the nucleotide triplets that code for amino acids 181 and 182 of that protein. This site was employed by S. Nagata et al., supra, in their synthesis of polypeptides displaying an immunological or biological activity of Le IF. One of the two HindII endonuclease recognition sites is between the triplets coding for amino acids 101 and 102 and one of the several Tag sites at the triplet coding for amino acid 45 of β-lactamase in pBR322. In similar fashion, the EcoRI site and the PvuII site in this plasmid lie outside of any coding region, the EcoRI site being located between the genes coding for resistance to tetracycline and ampicillin, respectively. This site was employed by T. Taniguchi, et al., supra, in their recombinant synthetic scheme. These sites are well recognized by those of

skill in the art. It is, of course, to be understood that a cloning vehicle useful in this invention need not have a restriction endonuclease site for insertion of the chosen DNA fragment. Instead, the vehicle could be joined to the fragment by alternative means.

The vector or cloning vehicle and in particular the site chosen therein for attachment of a selected DNA fragment to form a recombinant DNA molecule is determined by a variety of factors, e.g., number of sites susceptible to a particular restriction enzyme, size of the protein to be expressed, susceptibility of the desired protein to proteolytic degradation by host cell enzymes, contamination or binding of the protein to be expressed by host cell proteins difficult to remove during purification, expression characteristics, such as the location of start and stop codons relative to the vector sequences, and other factors recognized by those of skill in the art. The choice of a vector and an insertion site for a particular gene is determined by a balance of these factors, not all selections being equally effective for a given case.

Although several methods are known in the art for inserting foreign DNA into a cloning vehicle or vector to form a recombinant DNA molecule, the method preferred for initial cloning in accordance with this invention is characterized by digesting the plasmid (in particular pBR322) with that restriction enzyme specific to the site chosen for the insertion (in particular PstI) and adding dA tails to the 3' termini by terminal transferase. In similar fashion, the double-stranded cDNA is elongated by the addition of dT tails to the 3' termini to allow joining to the tailed plasmid. The tailed plasmid and cDNA are then annealed to insert the cDNA in the appropriate site of the plasmid and to circularize the hybrid DNA, the complementary character of the tails permitting their cohesion (Figure 1). The

resulting recombinant DNA molecule now carries an inserted gene at the chosen PstI restriction site (Figure 1). This method of dA-dT tailing for insertion is described by D. A. Jackson, et al., "Biochemical Methods For Inserting New Genetic Information Into DNA Of Simian Virus 40: Circular SV40 DNA Molecules Containing Lambda Phage Genes And The Galactose Operon Of Escherichia coli", Proc. Natl. Acad. Sci. USA, 69, pp. 2904-909 (1972) and R. Devos, et al., supra. It results in about 3 times as many recombinant DNA plasmids as dG-dC tailing.

Of course, other known methods of inserting DNA sequences into cloning vehicles to form recombinant DNA molecules are equally useful in this invention. These include, for example, dG-dC tailing, direct ligation, synthetic linkers, exonuclease and polymerase-linked repair reactions followed by ligation, or extension of the DNA strand with DNA polymerase and an appropriate single-stranded template followed by ligation.

It should also be understood that the nucleotide sequences or cDNA fragment inserted at the selected site of the cloning vehicle may include nucleotides which are not part of the actual structural gene for the desired polypeptide or mature protein or may include only a fragment of the complete structural gene for the desired protein.

The cloning vehicle or vector containing the foreign gene is employed to transform a host so as to permit that host to replicate the foreign gene and to express polypeptide(s) displaying an immunological or biological activity of human fibroblast interferon. The selection of an appropriate host is also controlled by a number of factors recognized by the art. These include, for example, compatibility with the chosen vector, toxicity of proteins encoded by the hybrid plasmid, ease of recovery of the desired protein, expression characteristics, bio-safety and costs. A balance of these

factors must be struck with the understanding that not all hosts may be equally effective for expression of a particular recombinant DNA molecule.

In the present synthesis, the preferred initial cloning vehicle is the bacterial plasmid pBR322 and the preferred initial restriction endonuclease site therein is the PstI site (Figure 1). The plasmid is a small (molecular weight approx. 2.6 megadaltons) plasmid carrying resistance genes to the antibiotics ampicillin (Amp) and tetracycline (Tet). The plasmid has been fully characterized (F. Bolivar, et al., "Construction And Characterization Of New Cloning Vehicles II. A Multi-Purpose Cloning System", Gene, pp. 95-113 (1977); J. G. Sutcliffe, "pBR322 Restriction Map Derived From The DNA Sequence: Accurate DNA Size Markers Up To 4361 Nucleotide Pairs Long", Nucleic Acids Research, 5, pp. 2721-28 (1978); J. G. Sutcliffe, "Complete Nucleotide Sequence Of The Escherichia coli Plasmid pBR322", Cold Spring Harbor Symposium, 43, I, pp. 77-90 (1978)). Insertion of the DNA product in this site provides a large number of bacterial clones each of which contains one of the DNA genes or fragments thereof present in the cDNA product previously prepared. Again, only a very few of these clones will contain the gene for F IF or fragments thereof (Figure 1) and none of them may permit the expression of polypeptide(s) displaying an immunological or biological activity of F IF. The preferred host in accordance with this invention is E. coli HB 101.

1. Preparation of PstI-Cleaved, dA-elongated pBR322

Plasmid pBR322 was digested completely at 37°C with PstI endonuclease (New England Biolabs) in 10 mM Tris-HCl (pH 7.6), 7 mM $MgCl_2$, 7 mM 2-mercaptoethanol. The mixture was extracted with 1 vol phenol and 10 vol ether and precipitated with 2.5 vol ethanol:0.2 M sodium acetate solution.

Addition of homopolymeric dA tails (Figure 1) by terminal deoxynucleotidyl transferase (TdT) (purified according to L. Chang and F. J. Bollum, "Deoxynucleotide-Polymerizing Enzymes Of Calf Thymus Gland", J. Biol. Chem., 246, pp. 909-16 (1971)) was done in a 50-µl reaction volume containing 0.14 M potassium cacodylate, 30 mM Tris-HCl (pH 6.8), 1 mM $CoSO_4$, 0.2 µg/µl heat-inactivated bovine serum albumin, 0.8 mM DTT, 0.2 mM dATP and some α ^{32}P-dATP. Incubation was at 37°C for 5 min before EDTA was added to 10 mM and SDS to 0.1% and the mixture extracted with phenol and chromatographed on Sephadex G50 in TE buffer. The void fractions, containing the linearized and elongated pBR322, were further purified by adsorption in 10 mM Tris-HCl (pH 7.8), 1 mM EDTA and 0.4 M NaCl to oligo(dT) cellulose. After extensive washing, the desired fractions were eluted with 10 mM Tris-HCl (pH 7.8) and 1 mM EDTA.

2. Preparation of dT-elongated DNA

Double-stranded DNA was elongated with dTMP residues in similar fashion to that described above for dA tailing of pBR322, except that dTTP and some ^{3}H-dTTP replaced the dATP and α ^{32}P-ATP. Purification on oligo(dT) cellulose was, of course, omitted. As before, the dT-elongated DNA is a mixture of different species, only a very few of which are F IF-related (Figure 1).

3. Preparation of Ca^{++}-Treated E. coli HB101

Ca^{++}-treated E. coli HB101 was prepared by the method of E. M. Lederberg and S. N. Cohen, "Transformation Of Salmonella Typhimurium By Plasmid Deoxyribonucleic Acid", J. Bacteriol., 119, pp. 1072-74 (1974) by inoculating the E. coli HB101 (a gift from H. Boyer) into 5 ml LB medium (10 parts bactotryptone, 5 parts yeast extract and 5 parts NaCl per liter) and cultures grown overnight at 37°C. The fresh cultures were diluted

None of the clones may permit the expression of polypeptides displaying an immunological or biological activity of F IF.

5. Transfection Of E. coli HB101 With The Annealed Hybrid Plasmids

P3 containment facilities were used as necessary for the transfection process and all subsequent steps in which the resulting transformed bacteria were handled. Aliquots (90 µl or less) of the above mixture were cooled to 0°C and 1 M $CaCl_2$ added to 0.1 M. Aliquots (100 µl or less) of this solution were added to 200 µl Ca^{++}-treated E. coli HB101 in ice and after standing at 0°C for 30 min, the cells were heat-shocked for 5 min at 37°C and cooled again at 0°C for 15 min. After addition of 2 ml LB-medium, the cells were incubated at 37°C in a shaking water bath for 30 to 45 min and the bacterial suspension plated out onto 1.2% agar plates, containing LB medium supplemented with 10 µg/ml tetracycline.

Since plasmid pBR322 includes the gene for tetracycline resistance, E. coli hosts which have been transformed with a plasmid having that gene intact will grow in cultures containing that antibiotic to the exclusion of those bacteria not so transformed. Therefore, growth in tetracycline-containing culture permits selection of hosts transformed with a recombinant DNA molecule or recyclized vector.

After 24 h at 37°C, individual colonies were picked and suspended in 100 µl LB medium (supplemented as above) in the wells of microtiter plates (Dynatech). After incubation at 37°C overnight, 11 µl dimethylsulfoxide were mixed into each well and the trays sealed with adhesive tape. The plates were stored at -20°C and a library of 17,000 individual clones of transformed E. coli HB101 was prepared. This library was derived from 270 fmoles (128 ng) dT-tailed cDNA inserts, which in

turn were synthesized from 4.4 µg gradient purified poly(A) RNA. About 98% of the clones of this library were sensitive to carbenicillin (a more stable ampicillin derivative). Therefore, about 98% of the library contained a plasmid having an insert in the PstI-site of the β-lactamase gene of pBR322 and only about 2% contained a recyclized vector without insert.

These 17,000 clones contain a variety of recombinant DNA molecules representing complete or partial copies of the mixture of mRNAs in the poly(A) RNA preparation from F IF-producing cells (Figure 2). The majority of these will contain only a single recombinant DNA molecule. Only a very few of these recombinant DNA molecules are related to F IF. Accordingly, the clones must be screened to separate the F IF-related clones from the others.

SCREENING FOR A CLONE CONTAINING F IFcDNA

There are several approaches to screen for bacterial clones containing F IFcDNA. These include, for example, RNA selection hybridization (Alwine, et al., infra), differential hybridization (T. F. St. John and R. W. Davis, "Isolation Of Galactose-Inducible DNA Sequences From Saccharomyces Cerevisiae By Differential Plaque Filter Hybridization", Cell, 16, pp. 443-452 (1979)); hybridization with a synthetic probe (B. Noyes, et al., "Detection And Partial Sequence Analysis Of Gastrin mRNA By Using An Oligodeoxynucleotide Probe", Proc. Natl. Acad. Sci. USA, 76, pp. 1770-74 (1979)) or screening for clones that produce the desired protein by immunological (L. Villa-Komaroff, et al., "A Bacterial Clone Synthesizing Proinsulin", Proc. Natl. Acad. Sci. USA, 75, pp. 3727-31 (1978)) or biological (A.C.Y. Chang, et al., "Phenotypic Expression In E. coli Of A DNA Sequence Coding For Mouse Dihydrofolate Reductase", Nature, 275, pp. 617-24 (1978)) assays. We have chosen

RNA selection hybridization as being the most convenient and promising method for primary screening.

A. RNA Selection Hybridization Assay

1. Overview Of The Initial Assay

Referring now to Figure 2, the recombinant DNA molecules were isolated from individual cultures of about 46 clones sensitive to carbenicillin and resistant to tetracycline from the above library of clones (two mixtures of 2 clones shown in Figure 2) (Step A). The recombinant DNA molecules were cleaved and hybridized to total RNA containing F IFmRNA prepared as before (Step B). All recombinant DNA molecule-total RNA hybrids were separated from the non-hybridized total RNA (Step C). The hybridized total RNA was recovered from the hybrids and purified (Step D). The recovered RNA was assayed for F IFmRNA activity as above (Step E). If, and only if, the mixture of recombinant DNA molecules contains a recombinant DNA molecule having an inserted nucleotide sequence capable of hybridizing to the F IFmRNA in the total RNA, under stringent hybridization conditions, will the mRNA released from that hybrid cause the formation of F IF in oocytes, because mRNA released from any other recombinant DNA molecule-total RNA hybrid will not be F IF-related. If a group of 46 clones gave a positive response, the clones were regrouped into 6 subgroups (4 subgroups of 8 and 2 subgroups of 7) and each subgroup assayed as before. This process was continued until a single clone responding to this assay was identified.

There is no assurance that the recombinant DNA molecules and bacterial cultures transformed therewith, which are thus identified, contain the complete F IFcDNA sequence of F IF or even that the DNA sequence actually codes for F IF or will permit the clone to express polypeptides displaying an immunological or biological

activity of F IF. However, the recombinant DNA molecules will certainly contain extensive nucleotide sequences complementary to the F IFmRNA coding sequence. Therefore, the recombinant DNA molecule may at least be used as a source of a probe to screen rapidly other recombinant DNA molecules and clones transformed with them to identify further sets of clones which may contain an authentic and complete F IF nucleotide coding sequence. These clones may then be analyzed for possible expression of polypeptides displaying a biological or immunological activity of F IF. The nucleotide sequence of the inserted DNA fragment of these hybrid plasmids and its amino acid translation product may also be determined and correlated to the amino acid composition and initial sequence reported for authentic F IF (supra).

2. Execution Of The Initial Assay

Step A - Preparation Of The Recombinant DNA Molecule Mixture

Replicas of a microtiter plate containing 96 clones from the above library of clones were made on LB-agar plates, one containing 10 µg/ml tetracycline and the other supplemented with 100 µg/ml carbenicillin. In this manner, two sets of about 45-46 clones, resistant to tetracycline and sensitive to carbenicillin, were picked and grown separately overnight at 37°C in 100 ml LB medium, containing 10 µg/ml tetracycline. These cultures were pooled, spun down in a Sorvall GS-3 rotor at 8000 rpm for 10 min, washed twice with TES buffer (50 mM Tris-HCl (pH 8), 5 mM EDTA, 5 mM NaCl) and resuspended in 60 ml TES per l of initial culture volume. The cells were lysed with lysozyme-Triton X-100 (M. Kahn, et al., "Plasmid Cloning Vehicles Derived From Plasmids Col E1, F, R6K And RK2" in Methods In Enzymology, 68: Recombinant DNA (R. Wu, ed.) (1980) (in press)). Forty ml of the TES suspended cells were combined with

20 ml 10% sucrose in 50 mM Tris-HCl (pH 8) and lysozyme to 1.2 mg/ml and allowed to stand at room temperature for 20 min. To this suspension were added 1 ml 0.5 M EDTA-NaOH (pH 8), 8 ml 0.2% Triton X-100, 25 mM EDTA, 50 mM Tris-HCl (pH 8) and the lysis completed at room temperature for 30 min. Cellular debris and most of the chromosomal DNA were removed by pelleting in a Beckman SW27 rotor at 24000 rpm for 45 min. The supernatant was cooled in ice, combined with 1/3 vol 40% polyethylene glycol 6000-2 M NaCl and allowed to stand overnight at 0°C. The resulting precipitate was collected in a Sorvall SB4 rotor at 5000 rpm for 10 min at 4°C and dissolved in TES buffer. The solution, with 0.2 vol 10 mg/ml ethidium bromide (Serva) and CsCl to 1 g/ml, was centrifuged in a Beckmann R60 Ti-rotor at 40000 rpm for at least 48 h, one polyallomer tube usually being sufficient for the lysate from 1-2 l of original culture volume. Two DNA bands could be visualized in the tube under UV-illumination. The band of highest density corresponds to plasmid form I DNA, the second band corresponds to form II and form III plasmid DNAs and some chromosomal DNA. The first band was collected from the tube, ethidium bromide removed by six isoamyl alcohol extractions, and the aqueous phase diluted with 3 vol water-supplemented with up to 0.2 M sodium acetate (pH 5.1) before DNA precipitation with 2.5 vol ethanol. The DNA was redissolved, extracted with phenol and again precipitated with ethanol. The quality of the DNA was monitored by electrophoresis on a 1% agarose gel in 40 mM Tris-HOAc (pH 7.8), 20 mM sodium acetate, 2 mM EDTA, followed by ethidium bromide staining. If the DNA was contaminated with too much RNA, it was further purified by neutral sucrose-gradient centrifugation: 300 µg DNA in 10 mM Tris-HCl (pH 7.6) and 1 mM EDTA were loaded on a 36-ml 5-20% sucrose gradient in 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 1 M NaCl, centrifuged in polyallomer

tubes for 16 h at 24000 rpm in a Beckmann SW27 rotor at 18°C and the DNA containing fractions (OD_{260}) pooled and precipitated with sodium acetate-ethanol.

Step B - Hybridization Of The DNA With Total RNA

About 150 µg DNA, thus prepared, were combined with some uniformly labelled ^{32}P-marker DNA and 2 µg pSTNV-1 DNA (a recombinant plasmid containing a full size cDNA copy of satellite tobacco necrosis virus ("STNV")-RNA; J. Van Emmelo, et al., "Construction And Characterization Of A Plasmid Containing A Nearly Full-Size DNA Copy Of Satellite Tobacco Necrosis Virus RNA", J. Mol. Biol., (in press) as internal control, sheared by sonication in an MSE sonicator and precipitated with sodium acetate-ethanol.

A diazobenzyloxymethyl (DBM)-cellulose solid matrix (Cf., J. C. Alwine, et al., "Method For Detection Of Specific RNAs In Agarose Gels By Transfer To Diazobenzyl Oxymethyl Paper And Hybridizing With DNA Probes", Proc. Natl. Acad. Sci. USA, 74, pp. 5350-54 (1977)) was prepared according to the method of J. C. Alwine, et al., "Detection Of Specific RNAs Or Specific Fragments Of DNA Fractionation In Gels And Transfer To Diazobenzyloxymethyl Paper", Methods-Enzymology, 68:Recombinant DNA (R. Wu, ed.) (1980) for a paper matrix, a sheet of Whatman 540 paper was evenly soaked in a solution containing 2-3 mg 1-(m-nitrobenzyloxy)methyl pyridinium chloride (NBPC/BDM and 0.7 ml sodium acetate trihydrate in 28.5 µl water per cm^2, incubated at 60°C until dry and for further 10 min, and baked at 130-135°C for 30-40 min. After washing several times with water (about 20 min), 3 times with acetone (about 20 min), and drying it was stored. The paper was incubated at 60°C for 30 min in 0.4 ml 20% sodium dithionite-water per cm^2 with occasional shaking. The paper was again washed four times with water, once

with 30% acetic acid for 5 min and four times with water, transferred to 0.3 ml per cm^2 ice-cold 1.2 N HCl to which 10 mg/ml fresh $NaNO_2$ had been added immediately before use for 30 min at 0°C, and washed twice quickly with ice-cold water and once with 80% dimethyl sulfoxide (spectrophotometric grade, Merck)-20% 25 mM sodium phosphate (pH 6.0). For a powder matrix essentially the same procedure was followed using microgranular cellulose powder (Whatman CC31), the quantities being expressed against the corresponding weight of the cellulose matrix.

Initially, we used a powder matrix because the capacity for binding was higher, so relatively smaller volumes for hybridization, washes and elution could be used. Subsequently we used a paper matrix for individual clone screening. Use of paper permits efficient elution with water which proved superior for the later assay of F IFmRNA.

The DNA prepared above was dissolved in 25 mM sodium phosphate (pH 6.0) heated for 1 min, chilled and four vol DMSO added. Coupling to the matrix (50 mg (powder) or a paper disc (10 mm dia.)) usually proceeded over a weekend at 4°C with continuous mixing. The volume of the DNA was kept rather small to allow close contact with the matrix and thereby enhance efficient coupling of the DNA to the matrix. After coupling, the matrix was washed four times with water and four times with 0.4 N NaOH at 17°C for 10 min each, again four times with water at room temperature and finally twice with hybridization buffer (50% formamide (deionized, Baker), 40 mM piperazine-N,N'-bis(2-ethane sulfonic acid) (pH 6.4) ("PIPES, Sigma), 1 mM EDTA, 0.6 M NaCl and 0.1% SDS) at 4°C. Coupling efficiencies were measured by ^{32}P-radioactivity.

Twenty µg total RNA, prepared as before, and 50 ng ETHV-RNA were dissolved in 250 µl (50 µl for paper matrix) hybridization buffer and added to the DNA coupled

matrix. The matrix was heated to 70°C for 2 min and held at 37°C overnight with gentle mixing.

Step C - Separation Of Hybridized Total RNA-DNA From Non-Hybridized Total RNA

After centrifugation of the powder matrix, the unhybridized RNAs were removed and the matrix washed seven times with a total 2 ml 50% formamide, 10 mM PIPES (pH 6.4), 1 mM EDTA, 0.3 M NaCl and 0.1% SDS, the lower salt content of these washes destabilizing non-specific RNA-DNA binding. Each wash was followed by centrifugation and resuspension of the matrix in the buffer. For subsequent assay, the first wash was pooled with the unhybridized RNA ("Fraction 1") and washes 2-4 ("Fraction 2") and washes 5-7 ("Fraction 3") were pooled. In hybridizations to a paper matrix, a similar procedure was utilized except that the total wash volume was limited 1 ml.

Step D - Purification Of Hybridized Total RNA

The hybridized total RNA-DNA was eluted from a powder matrix with 3 elutions of a total 900 µl 99% formamide, 0.2% SDS at 70°C for 2 min and chilled in ice. The total hybridization procedure and elution with formamide were essentially as described by A. G. Smith, personal communication. The hybridized total RNA-DNA was eluted from a paper matrix by first washing with 100 µl of ice cold water and following that with two water elutions (total 300 µl) at 80°C for 2 min. For subsequent assay these elutions and the 100 µl wash were pooled ("Fraction 4").

To one-half of each fraction, 0.1 µg calf liver tRNA or ribosomal RNA were added (Fractions 1A, 2A, 3A and 4A) and to the other half 8 µg eukaryotic poly(A) RNA or ribosomal RNA were added (Fractions 1B,

2B, 3B, 4B). The fractions were purified by precipitation by the addition of 0.5 M NaCl and 2.5 vol ethanol to removal traces of formamide and other impurities.

Step F - Determination Of F IFmRNA Activity

Fractions 1A, 2A, 3A and 4A were translated in 25 µl nuclease-treated rabbit reticulocyte lysate (prepared according to the procedure of R. E. Pelham and R. J. Jackson, "An Efficient mRNA-Dependent Translation System For Reticulocyte Lysates", Eur. J. Biochem., 7, pp. 247-56 (1976)) by the procedure of B. LeBleu, et al., "Translation Of Mouse Interferon mRNA In Xenopus Laevis Oocytes And In Rabbit Reticulocyte Lysates", Biochem. Biophys. Res. Commun., 82, pp. 665-673 (1978) except that 250 mM spermidine-HCl, 1 mM fructose-1,6-diphosphate were added in the presence of ^{35}S-methionine (0.5 mCi/ml, Amersham). After incubation, 25 µl reticulocyte lysate, from above, were combined with 1 µl 10% deoxycholate-10% Triton X100 and 2 µl antiserum-PBS (1:9) and heated at 37°C for 1 h. Twenty µl Staphylococcus aureus Cowan I (freshly washed, S. W. Kessler, et al., "Rapid Isolation Of Antigens From Cells With A Staphylococcal Protein A-Antibody Adsorbent: Parameters Of The Interaction Of Antibody-Antigen Complexes With Protein A", J. Immunology, 115, pp. 1617-1624 (1975) in 10% 100 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.05% NP40 were added and the mixture maintained at 20°C for 30 min and centrifuged in an Eppendorf 5412 centrifuge for 2 min. The pellet was washed and centrifuged twice with PBS and the final pellet dissolved in sample buffer and electrophoresed in 13% polyacrylamide gel as described by U. K. Laemmli, et al., "Cleavage Of Structural Proteins During The Assembly Of The Head Of Bacteriophage T4", Nature, 227, pp. 680-85 (1970), and autoradiographed. Comparison of the STMV-RNA translation products in Fractions 1A and

4A provide an indication of the efficiency of hybridization and RNA degradation in the process.

Fractions 1B, 2B, 3B and 4B were dissolved in 2 µl water and assayed in oocytes for F IFNRNA content as described above.

3. Subsequent Assay - Hybridization To Nitrocellulose Sheets

Some subsequent assays of individual clones were done on nitrocellulose sheets (M. Cochet, et al., "Cloning Of An Almost Full-Length Chicken Conalbumin Double-Stranded cDNA", Nucleic Acids Research, 6, pp. 2435-2452 (1979)). The DNA was dissolved in 2M NaCl and 0.2 M NaOH, heated at 100°C for 1 min, chilled, and spotted on detergent free Millipore filters (pore size 0.45 um; 7 mm dia.). The filters were baked for 2 h at 80°C, washed in 0.3 M NaCl, 2 mM EDTA, 0.1% SDS, 10 mM Tris-HCl (pH 7.5) and dried at room temperature. The RNA was hybridized for 3 h at 47°C in 30% formamide, 0.5 M NaCl, 0.4% SDS, 2 mM EDTA, 50 mM PIPES (pH 7.5). Hybridization was stopped by dilution with 10 vol 0.1 M NaCl and the filters were washed several times in 15 ml 0.3 M NaCl, 0.1% SDS, 2 mM EDTA, 10 mM Tris-HCl (pH 7.5) by shaking at 45°C and several times in the same solution without SDS at 4°C. Elution of the hybridized RNA-DNA was effected in 30 µl 5 mM potassium chloride at 100°C for 1 min.

4. Results Of The RNA Selection Hybridization Assay

Sixteen groups of about 46 clones were screened (Groups A-P). In six of the groups, Fraction 1B contained the only F IFmRNA activity, in eight of the groups no F IFmRNA was detected and in two groups (Groups C and O) F IFmRNA was observed in Fraction 4B. The group C and O assays are reported in the following format: logarithm of F IF units (calibrated against reference standard 69/19), detected in the assay of Fraction 1B (non-hybridized) and in the assay of Fraction 4B (hybridized). The limit of detection was 0.1.

Group	Fraction 1B	Fraction 4B
C	1.0	0
	0.5	0.5
	0	0.2
O	0	0
	0.2	0.5

Group O was subdivided into 6 subgroups (Subgroups O_1 to O_6; four of eight clones and two of seven clones) and hybridized and assayed as before, except that a 400 ml culture per clone was used. The subgroups gave the following results, presented in the same format as above. Hybridization was carried out on DNB-cellulose powder except as otherwise indicated.

Subgroup	Fraction 1B	Fraction 4B
O_1	0	1.2
	0	1.5
	0	0.5
	0	0.5
	0.2	0.5
	0	1.2*
O_2	0.7	0
O_3	0.7	0
	0.5	0
O_4	0	0
O_5	0.5	0
O_6	0	0

Subgroup O_1 was subdivided into its individual clones (designated clones $O_{1/1}$ - $O_{1/8}$) and hybridized and assayed as before, except that a 700 ml culture per clone was used. The hybridization was again carried out on DBM-cellulose powder except as otherwise indicated

Clone	Fraction 1B	Fraction 4B
$O_{1/1}$	0.2	0
	0.7	0
	0.7	0*
	1.0	0**
$O_{1/2}$	1.2	0
	0.2	0*
	0.7	0**
$O_{1/3}$	1.2	0
	1.0	0.2*
	1.2	1.0(?)*
	1.2	0**

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* DBM cellulose paper method.

** Nitrocellulose sheets

$O_{1/4}$	1.2	0
	1.2	0
	1.0	0*
	1.2	0**
$O_{1/5}$	0.7	0
	0.7	≤0.2*
	1.0	0
$O_{1/6}$	0.7	0
	1.0	≤0.2*
	0.5	0**
$O_{1/7}$	0.5	0
	1.2	0*
	≤0.2	0.5**
$O_{1/8}$	0	1.7*
	≤0.2	1.2*
	0	0.7**
	0	1.0**

Therefore, clone 01/8 contains a recombinant DNA molecule capable of hybridizing F IF mRNA from total RNA containing F IF mRNA. Non-specific RNA-DNA binding is highly unlikely, because a comparison of Fractions 1A and 4A revealed substantially no non-specific binding of STNV DNA in these same experiments. *i.d.*, as monitored by translation in a rabbit reticulocyte lysate in the presence of 35S-methionine, followed by gel electrophoresis, as described above. Clone 01/8 was designated *E. coli* HB101 (G-pBR322(Pst)/HFIF1 ("G-HB101-pHFIF1"), its recombinant DNA molecule G-pBR322 (Pst)HcFIF1 ("pHFIF1") and its hybrid insert "pHFIF1 fragment". This nomenclature indicates that the clone and recombinant DNA molecule originated in Ghent ("G") and comprises plasmid pBR322 containing, at the PstI site HFIF cDNA ("HFIF"), the particular molecule being the first located.

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* DBM cellulose paper method

** Nitrocellulose sheets

IDENTIFICATION OF CLONES CONTAINING RECOMBINANT DNA-MOLECULES CROSS-HYBRIDIZING TO pHFIF1

pHFIF1, isolated above, was used to screen the library of clones, prepared previously, for bacterial clones containing recombinant DNA molecules having related hybrid DNA inserts, by colony hybridization (M. Grunstein and D. S. Hogness, "A Method For The Isolation Of Cloned DNA's That Contain A Specific Gene", Proc. Natl. Acad. Sci. USA, 72, pp. 3961-3965 (1975)). This method allows rapid identification of related clones by hybridization of a radioactive probe to the DNA of lysed bacterial colonies fixed in nitrocellulose filters.

The library of clones stored in microtiter plates as described above, was replicated on similar size nitrocellulose sheets (0.45 µm pore-diameter, Schleicher and Schuell or Millipore), which had been previously boiled to remove detergent, and the sheets placed on LB-agar plates, containing tetracycline at 10 µg/ml. Bacterial colonies were grown overnight at 37°C. Lysis and fixation of the bacteria on the nitrocellulose sheets took place by washing consecutively in 0.5 N NaOH (twice for 7 min.), 1 M Tris-HCl (pH 7.5) (7 min.), 0.5 M Tris-HCl (pH 7.5) and 1.5 M NaCl (7 min.), 2 x SSC (0.15 M NaCl, 0.015 M sodium citrate (pH 7.2) (for 7 min.)). After thorough rinsing with ethanol and air drying, the sheets were baked at 80°C for 2 h in vacuo and stored at room temperature.

A Hinf I restriction fragment specific for the pHFIF1 fragment (infra) served as the probe for colony hybridization, described infra. This fragment (~170 basepairs) was purified by electrophoresis of the Hinf digestion products of pHFIF1 in a 6% polyacrylamide gel. After staining the DNA bands with ethidiumbromide, the specific fragment was eluted, reelectrophoresed and ^{32}P-labelled by "nick translation" (P.W.J. Rigby et al.,

"Labeling Deoxyribonucleic Acid To High Specific Activity In Vitro By Nick Translation With DNA Polymerase I", J. Mol. Biol., 113, pp. 237-251 (1977)) by incubation in 50 l 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 20 mM β-mercaptoethanol, containing 2.5 µl each of dCTP, dTTP and dGTP at 400 µM, 100 pmoles α-^{32}P-ATP (Amersham, 2000 Ci/mmole) and 2.5 units of DNA-polymerase I (Boehringer) at 14°C for 45 min. The unreacted deoxynucleoside triphosphates were removed by gel filtration over Sephadex G-50 in T.E. buffer. The highly ^{32}P-labelled DNA was precipitated with 0.1 vol of 2 M sodium acetate (pH 5.1) and 2.5 vol of ethanol at 20°C.

Hybridization of the above probe to the filter impregnated DNA was carried out essentially as described by D. Hanahan and M. Meselson (personal communication): The filters, prepared above, were preincubated for 2 h at 68°C in 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, 0.15 M NaCl, 0.03 M Tris-HCl (pH 8), 1 mM EDTA, and rinsed with 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 0.75 M NaCl, 0.15 M Tris-HCl (pH 8), 5 mM EDTA and 0.5% SDS. The hybridization proceeded overnight at 68°C in a solution identical to the rinsing solution above using the ^{32}P-labelled probe which had been denatured at 100°C for 5 min prior to use. The hybridized filters were washed twice with 0.3 M NaCl, 0.06 M Tris-HCl (pH 8), 2 mM EDTA for 2 h at 68°C before air drying and autoradiography.

About 1350 clones, originating from the 800-900 DNA size class, were screened. Thirteen colonies, including pHFIF1, gave a positive result. These clones were designated G-HB101-pHFIF1 to 13 and their recombinant DNA molecules pHFIF1 to 13. One of the clones, pHFIF2, was hybridized with poly(A) mRNA containing F IF mRNA and assayed using DBM-cellulose paper (supra). Because the total IF-RNA activity was detected in the hybridized

fraction and the unhybridized RNA did not contain any detectable activity, it is clear that clones identified by colony hybridization to a part of the pHFIF1 fragment also hybridized to F IF mRNA.

It is, of course, evident that this method of clone screening may be employed equally well on other clones containing DNA sequences arising from recombinant DNA technology, synthesis, natural sources or a combination thereof or clones containing DNA sequences related to any of the above DNA sequences by mutation, including single or multiple, base substitutions, insertions, inversions, or deletions. Therefore, such DNA sequences and their identification also fall within this invention. It is also to be understood that DNA sequences, which are not screened by the above DNA sequences, yet which as a result of their arrangement of nucleotides code for those polypeptides coded for by the above DNA sequences also fall within this invention.

CHARACTERIZATION OF THE F IF-RELATED RECOMBINANT PLASMIDS

The thirteen clones which were detected by colony hybridization were further characterized. A physical map of the inserts of these clones was contructed and the orientation of the inserts in the various clones was determined.

The physical maps of the plasmids were constructed by digestion with various restriction enzymes (New England Biolabs) in 10 mM Tris-HCl (pH 7.6), 7 mM $MgCl_2$ and 7 mM β-mercaptoethanol at 37°C by well-known procedures. The products of digestion were electrophoresed in 2.2% agarose or 6% polyacrylamide gels in 40 mM Tris-HOAc (pH 7.6), 20 mM EDTA. They were analyzed after visualization by staining with ethidiumbromide and compared with the detailed physical map of pBR322 (J.G. Sutcliffe, supra). Restriction maps of the different plasmids were contructed on the basis of these digestion

patterns. These were refined by sequencing the DNA inserts in various of the plasmids, substantially by the procedure of A.M. Maxam and W. Gilbert, "A New Method for Sequencing DNA", Proc. Natl. Acad. Sci. USA, 74, pp. 560-564 (1977).

Plasmid DNA was prepared from various of the pHFIF1-13 in accordance with this invention by the method of Kahn et al. (supra), employed previously herein to isolate the DNA from the sets of clones for screening. The isolated form I DNA was purified by neutral sucrose-gradient centrifugation as before and restricted by various restriction enzymes, essentially as recommended by the supplier (New England Biolabs).

Restricted DNA was dephosphorylated for 30 min at 65°C in the presence of 4 units bacterial alkaline phosphatase and 0.1% SDS. Following two phenol extractions and ethanol precipitation, the DNA was 5'-terminally labelled with γ-^{32}P-ATP (~ 3000 Ci/mmole) and polynucleotide kinase (P-L Biochemicals, Inc.).

For sequencing, labelled fragments were handled in two ways. Some were purified on a polyacrylamide gel prior to cleavage with a second restriction enzyme. Others were immediately cleaved with a second restriction enzyme. In both cases the desired fragments were separated on a polyacrylamide gel in Tris-borate-EDTA buffer. Figure 7 displays the various restriction fragments (the circles indicating the label and the arrow the direction of sequencing) and the sequencing strategy employed using pHFIF1, pHFIF3, pHFIF6 and pHFIF7.

The fragments were degraded according to the method of A.M. Maxam and W. Gilbert (supra). The products were fractionated on polyacrylamide gels of various concentrations and lengths in 50 mM Tris-borate, 1 mM EDTA (pH 8.3) at 900 V to 2000 V.

Each stretch of cDNA insert was sequenced from both strands and each restriction site which served as

labelled terminus was sequenced using a fragment spanning it. The composite nucleotide sequence thus obtained for the coding strand of F IF DNA or gene and its corresponding amino acid sequence is depicted in Fig. 4. Because none of plasmids pHFIF1-13 contained the complete gene for fibroblast interferon, Fig. 4 results from a combination of the data from at least two such plasmids. In this regard, Fig. 5 displays the relationship of inserts pHFIF1, pHFIF3, pHFIF6 and pHFIF7, the solid arrows or chevrons indicating the orientation of the various parts of the inserts.

Referring now to Fig. 4, the heteropolymeric part of the insert is flanked on one end by a segment rich in T's and by a string of A's (probably reflecting the polyA terminus of the mRNA). For reference the insert is numbered from first nucleotide of the composite insert to a nucleotide well into the untranslated section of the insert. An ATG initiation triplet at position 65-67 and a TGA termination triplet at position 626-628 define a reading frame uninterrupted by nonsense codons. Any other translatable sequence, i.e., in different reading frames, flanked by an ATG or a GTG and a termination signal is too short to code for a polypeptide of the expected size of F IF. Therefore, the region between nucleotides 65 and 625 most likely includes the nucleotide sequence for the composite gene that codes for F IF in accordance with this invention. This sequence does not exclude the possibility that modifications to the gene such as mutations, including single or multiple, base substitutions, deletions, insertions, or inversions may not have already occurred in the gene or may not be employed subsequently to modify its properties or the properties of the polypeptides translated therefrom. Nor does it exclude any polymophism which may result in physiologically similar but structurally slightly different genes as polypeptides than that reported in

Figure 4, supra, p. 4. For example, another clone identified in accordance with this invention has a "T" instead of a 'C" at nucleotide 90 of the nucleotide sequence coding for F IF. This change in the third nucleotide of the codes does not change the amino acid and coded therefrom. The translated insert of this clone is identical in nucleotide sequence to that imported by Taniguichi et al., supra.

It should of course be understood that cloned cDNA from polyA RNA by the usual procedures (A. Efstratiadis et al, supra) lacks 5'-terminal nucleotides and may even contain artifactual sequences (R.I. Richards et al., "Molecular Cloning And Sequence Analysis Of Adult Chicken β-Globin cDNA", Nucleic Acids Research, 7, pp. 1137-46 (1979)). Therefore, it is not certain that the ATG located at nucleotides 65-67 is in fact the first ATG of authentic mRNA. However, for the purposes of the following description, it is assumed that the ATG at nucleotides 65-67 is the first ATG of authentic F IF DNA.

By comparing the polypeptide coded by this region of the insert with that sequence of 13 amino-terminal amino acids of authentic human fibroblast interferon -- MetSerTyr AsnLeuLeuGlyPheLeuGlnArgSerSer -- determined by Knight et al. (supra), it appears that the chosen reading frame is correct and that nucleotides 65-127 may code for a signal sequence which precedes the nucleotide sequence coding for the "mature" polypeptide. In addition, in eukaryotic mRNAs the first AUG triplet from the 5' terminus is usually the initiation site for protein synthesis (M. Kozak, How Do Eukaryotic Ribosomes Select Initiation Regions In Messenger RNA?", Cell, 15, pp. 1109-25 (1978)). Here, the codon in the composite fragment corresponding to the first amino acid of fibroblast interferon is 22 codons from the first ATG. This again suggests that the DNA sequence coding for fibroblast

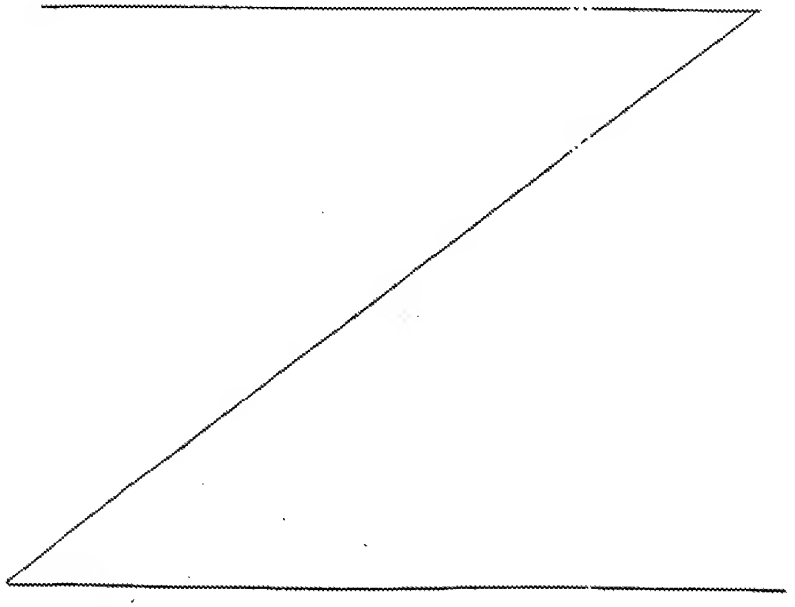
interferon may be preceded by a sequence determining a signal polypeptide of 21 amino acids. The presumptive signal sequence contains a series of hydrophobic amino acids. An accumulation of hydrophobic residues is characteristic of signal sequences (c.f., B.D. Davis and P.C. Tai, "The Mechanism Of Protein Secretion Across Membranes", Nature, 283, pp. 433-38 (1980)).

The nucleotide sequence apparently corresponding to "mature" F IF polypeptide comprises 498 nucleotides, which code for 166 amino acids. Assuming that there is no carboxyterminal processing, the molecular weight of the interferon polypeptide is 20085. The base composition of the coding sequence is 45% G+C. The codon usage within the interferon coding sequences is in reasonable agreement with that complied for mammalian mRNAs in general (R. Grantham et al., "Coding Catalog Usage And The Genome Hypothesis", Nucleic Acids Research, 8, pp. 49-62 (1980)). Any deviations observed may be ascribed to the small numbers involved.

The structure of the polypeptide depicted in Fig. 4 for the composite fragment, of course, does not take into account any modifications to the polypeptide caused by its interaction with in vivo enzymes, e.g., glycosylation. Therefore, it must be understood that the amino acid sequence depicted in Figure 4 may not be identical with F IF produced in vivo.

The comparison of the first 13 amino acids of authentic fibroblast interferon (Knight et al., supra) and the sequence deduced from the composite gene of Fig. 4 shows no differences. The amino acid compositions determined directly for authentic fibroblast interferon on the one hand and that deduced from the sequence of the composite gene of this invention on the other also show substantial similarities. Fig. 6 displays a comparison of these compositions.

Although none of the recombinant DNA molecules prepared in accordance with this invention contain the complete DNA sequence for fibroblast interferon, a combination of portions of the inserts of these recombinant DNA molecules to afford the complete F IF DNA gene sequence is within the skill of the art. For example, by reference to Fig. 5, it can readily be seen that the PstI-BclI fragment of pHF1F6 could be joined with the PstI-HaeII fragment of pHF1F7 or the EcoRI-PstI fragment of pHF1F6 could be joined with the PstI-HaeII fragment of pHF1F7 or the BclII-PstI fragment of pHF1F6 could be joined with the PstI-BclI fragment of clone 7 to form the composite F IF gene. The joining of those fragments could be done before or after insertion into a desired plasmid.



PREPARATION OF PLASMIDS CONTAINING THE COMPLETE HFIP GENE FOR THE PURPOSE OF EXPRESSING POLYPEPTIDES DISPLAYING HFIP ACTIVITY

Bacteriophage λ contains two strong promoters, P_L and P_R, whose activity is under the control of a repressor protein, the product of the phage gene cI. In the presence of repressor, transcription from these promoters is fully repressed. Removal of repressor turns on strong transcription from P_L and P_R (for review, see H. Szybalski and W. Szybalski "A Comprehensive Molecular Map Of Bacteriophage λ", Gene, 7, 217-270 (1979)).

Derivatives of the multicopy plasmid pBR322 (F. Bolivar et al. "Construction And Characterization Of New Cloning Vehicles. II. A Multiple Cloning System", Gene, 2, 95-113 (1977)) were constructed that incorporate the P_L promoter.

A. Structure Of Plasmids Containing The P_L Promoter

Plasmid pPLa2311

Plasmid pPLa2311 (shown in Fig. 8) consists of three Hae II fragments. The largest fragment, about 1940 base pairs, contains the P_LO_L region from bacteriophage λ and the β-lactamase gene region from pBR322 (F. Sutcliffe "Complete Nucleotide Sequence Of The Escherichia coli Plasmid pBR322", Cold Spring Harbor Symposia, 49, 77-90, (1978)). Adjacent to this fragment is a 370 base pairs Hae II fragment derived from plasmid $ColE_1$. The origin of replication spans the junction between these two fragments (A. Oka et al. "Nucleotide Sequence Of Small $ColE_1$ Derivatives. Structure Of The Regions Essential For Autonomous Replication And Colicin E_1 Immunity" Mol. Gen. Genet., 172, 151-159 (1979)). The third Hae II fragment, about

1600 base pairs in length, codes for resistance to kanamycin. This fragment was originally derived from plasmid pCR_1 (C. Covey et al. "A Method For The Detection Of Restriction Sites In Bacterial Plasmid DNA", Mol. Gen. Genet. 145, 155-158 (1976)). The direction of transcription from the P_L promoter runs in the same sense as the β-lactamase gene. Plasmid pPLa2311 confers resistance to 100 µg/ml carbenicillin and 50 µg/ml kanamycin.

Plasmid G-pPLa8

Plasmid G-pPLa8 (shown in Fig. 9) was derived from pPLa2311 by converting the PstI site in the β-lactamase gene to a BamHI site. This was accomplished by S_1 nuclease treatment of PstI-opened pPLa2311 followed by blunt end ligation to a BamHI linker fragment (obtained from Collaborative Research Inc., Waltham, Mass. cat. n°. 18029) and recirculalization of the molecule after BamHI cleavage. Plasmid pPLa8 no longer specifies resistance to carbenicillin.

Plasmid G-pPLc24

Plasmid G-pPLc24 (shown in Fig. 10) contains the β-lactamase gene and the origin of replication from pBR322. A 290 base pair HaeII-EcoRI fragment contains the $P_L O_L$ region from bacteriophage λ. The direction of transcription from the P_L promoter is towards the EcoRI site. A 431 base pair EcoRI-BamHI fragment codes for the ribosome binding site and the first 98 amino acid residues of the bacteriophage MS2 replicase gene, obtained from plasmid pMS2-7 (R. Devos et al. "Construction And Characterization Of A Plasmid Containing A Nearly Full-size DNA Copy Of Bacteriophage MS2 RNA" J. Mol. Biol. 128, 595-619 (1979)). Translation of the MS2 replicase protein fragment runs colinear with the transcription from the P_L promoter.

B. Temperature-dependent Switch-On Of P_L Promoter Activity

Transcription from the P_L promoter - present on plasmids pPLa2311, pPLa8 and pPLc24 - is repressed by maintaining the plasmids in an E. coli strain that synthesizes the repressor protein. Due to its autoregulating mode of synthesis (M. Ptashne et al. "Autoregulation And Function Of A Repressor In Bacteriophage λ", Science, 194, 156-161 (1976)), one copy of the cI gene on the chromosome of a lysogenic strain is able to fully repress the P_L promoter present on a multicopy plasmid.

The strains used were K12ΔHI (K12 M72 lac_{am} ΔtrpEA2 Sm^R (λcI857 $N_{am7}N_{am53}$ ΔHI bio^-); U. Bernard et al. "Construction Of Plasmid Cloning Vehicles That Promote Gene Expression From The Bacteriophage λ P_L Promoter" Gene, 5, 59-76 (1979)) and M5219 (K12 M72 lac_{am} trp_{am} Sm^R (λcI857 ΔHI bio252); N. Greer, "The kil Gene Of Bacteriophage λ" Virology, 66, 589-604 (1975)). Both strains harbor a defective, non-excisable λ prophage carrying a mutant cI gene. The mutant gene codes for a temperature-sensitive repressor, thus allowing to turn on transcription from the P_L promoter by shifting the temperature: at 28°C the repressor is active and represses transcription but at 42°C the repressor is inactivated and transcription from the P_L promoter is fully turned on.

The ΔHI deletion of the prophage removes part of the cro gene and all other genes further to the right of cro (M. Castellazzi et al. "Isolation And Characterization Of Deletions In Bacteriophage λ Residing As Prophage In E. coli K12" Mol. Gen. Genet. 117, 211-218 (1972)). The deletion of the cro gene is advantageous because accumulation of the cro protein is known to repress transcription from the P_L promoter (A. Johnson et al. "Mechanism Of Action Of The cro Protein Of Bacteriophage λ" Proc. Natl. Acad. Sci.

U.S.A. 75, 1783-1787, (1978)). Strain M5219 in addition contains the bio252 deletion which removes all genes to the left of cIII, including kil.

Upon temperature induction strain M5219 expresses a functional N-gene product. Strain K12ΔHI on the other hand has two amber mutations in N rendering it functionally N-negative. The product of the N gene is known to act as an anti-terminator in bacteriophage λ (J. W. Roberts, "Transcription Termination And Late Control In Phage λ" Proc. Natl. Acad. Sci. U.S.A. 72, 3300-3304 (1975)). The anti-termination effect was equally observed with terminator sequences not naturally present on phage λ DNA (e.g., the natural stop at the end of the trp operon), provided the RNA transcript starts at the P_L promoter. Furthermore, polarity effects, introduced by the presence of a nonsense codon in the P_L transcript, were relieved under the action of the N-gene protein (for review see N. Franklin and C. Yanofsky, "The N Protein Of λ: Evidence Bearing On Transcription Termination, Polarity And The Alteration Of E. coli RNA Polymerase" in RNA Polymerase (Cold Spring Harbor Laboratory, 1976) pp. 693-706).

Having the aforementioned plasmids in a thermoinducible bacterial cI background allows experimental switching on or off of the activity of P_L promoter. The choice of K12ΔHI or M5219 allows transcription to proceed either in the absence or presence of the N-gene product. The anti-termination properties of N could be advantageous in such instances where DNA regions are to be transcribed that contain transcription terminator-like sequences or slow-down sequences for the RNA polymerase.

C. Construction Of Clones Which Have The HFIF Gene Inserted Into A Plasmid Containing The P_L Promoter

Isolation of plasmid DNA, restriction analysis of DNA and ligation of DNA fragments were performed as described above in the cloning of double-stranded DNA. The transformation step was also as described above except that, when strains K12ΔHI or M5219 where used as the host, heat shock was done at 34°C for 5 min and the transformed cells were incubated at 28°C.

1. Construction Of Plasmid G-pPLa-HFIF-67-1

The rationale for this construction was the observation that combination of the appropriate restriction fragments from clones G-pBR322(Pst)/HFIF-6 and G-pBR322(Pst)/HFIF-7 allows the reconstruction of a complete, continuous coding sequence of the HFIF gene. The flow of the derived fragments through the several construction steps is shown schematically in Fig. 8. G-pBR322(Pst)/HFIF-6 DNA was cleaved with EcoRI and PstI and ligated to G-pBR322(Pst)/HFIF-7 DNA which had been cleaved with PstI and PvuI. Following ligation the mixture was digested with EcoRI and HaeII. A 4-fold molar excess of this mixture was then ligated to G-pPLa2311 DNA which had been digested with HaeII and EcoRI. Transformants were obtained in strain C600$r_K^-m_K^+$(λ), which was used because of its relatively high transformation capability and because it contains a wild-type cI gene, by selection for kanamycin resistance. Of 15 transformants screened, two had lost resistance to carbenicillin. Restriction analysis of isolated DNA revealed that one of these had the desired structure of G-pPLa-HFIF-67-1 depicted in Fig. 8. This plasmid contained a unique EcoRI site and a unique PstI site. Combined EcoRI-PstI digestion produced two fragments the smaller of which comigrated with a fragment obtained after EcoRI-PstI cleavage of G-pBR322(Pst)/

HFIF-6. BglII digestion cleaved out a small fragment of about 650 base pairs. The size of the latter fragment is consistent with the expected size after joining the proximal BglII-PstI fragment of clone G-pBR322(Pst)/HFIF-6 to the distal PstI-BglII part of G-pBR322(Pst)/HFIF-7. HincII digestion produced three fragments as expected from the presence of HincII sites on the P_L region, the amino-terminal part of the β-lactamase gene and the untranslated 5' end of the HFIF gene. This plasmid was designated G-pPLa-HFIF-67-1. Based on the aforementioned characterization by restriction enzyme analysis, plasmid G-pPLa-HFIF-67-1 should contain the complete coding sequence of the HFIF gene. The direction of desired transcription runs colinear with that from the P_L promoter. In between the P_L and the HFIF gene the plasmid still retains the poly(A·T) tail and an inverted 3' end fragment as in G-pBR322(Pst)/HFIF-6.

2. Construction Of Plasmid G-pPLa-HFIF-67-12

The next step in the constructions was aimed at removing from G-pPLa-HFIF-67-1 the poly(A·T) tail and part of the inverted 3' end fragment. G-pPLa-HFIF-67-1 DNA was cleaved with BglII and HpaII. Since the HFIF sequence contains no HpaII site this treatment results in the BglII fragment containing the entire coding sequence for HFIF and at the same time inactivates the remaining part of the vector. The DNA was ligated to G-pPLa8 DNA which had been digested with BamHI. The enzymes BglII and BamHI make identical staggered ends such that BglII ends can be ligated to an opened BamHI site and vice versa. Such a reconstructed site is no longer a substrate for BglII or BamHI enzyme action but is recognized by the enzyme Sau3AI (XhoI) (V. Pirotta, "Two Restriction Endonucleases From Bacillus globigii" Nucleic Acids Res. 3, 1747-1760 (1976)).

Following ligation the mixture was again cleaved with BamHI to eliminate these G-pPLa8 molecules that had simply recircularized. Transformants were obtained in C600$r_K^-m_K^+$(λ) selecting for kanamycin resistance. The transformants were screened by size determination of uncleaved DNA on agarose gel as described above for characterization of the HFIF-related recombinant plasmids. Clones which proved slightly larger than the G-pPLa8 parent were further subjected to restriction analysis with either PstI or HincII. One clone was found which contained a single PstI site and three HincII sites. One fragment comigrated with a HincII fragment from pPLa8 derived from the P_L to the β-lactamase region. Another small fragment measured about 400 base pairs, consistent with insertion of the BglII fragment into G-pPLa8 in the sense orientation with respect to the P_L promoter. This plasmid was designated G-pPLa-HFIF-67-12. The steps used in the construction of this plasmid are shown schematically in Fig. 9. A more detailed map of this plasmid is shown in Fig. 11. The size of the plasmid (~4450 base pairs) was estimated by the size of its constituent fragments, which in turn had been estimated by their relative mobility upon electrophoresis in agarose gels.

E. coli K12:HI and M5219 were then transformed with the characterized plasmid G-pPLa-HFIF-67-12.

Inspection of the known nucleotide sequence around the BglII/BamHI junction in G-pPLa-HFIF-67-12 revealed an interesting feature. Predictably, a polypeptide initiated at the AUG of the β-lactamase gene will terminate on a double amber codon within the untranslated 5'-end of the HFIF gene, as cloned in pPLa-HFIF-67-12, at 23 nucleotides before the initiating AUG. The predicted sequence around the fusion point reads:

* Junction
191 BamHI/BglII

CCC.CGG.ATC.TTC.AGT.TTC.GGA.GGC.AAC.CTT.TCG.AAG.CCT.TTG.CTC.
Pro-Arg-Ile-Phe-Ser-Phe-Gly-Gly-Asn-Leu-Ser-Lys-Pro-Leu-Leu-

TGG.CAC.AAC.AGG.TAG.TAG GCGACACTGTTCGTGTTGTCAAC AUG—HFIF coding sequence
Trp-His-Asn-Arg am am

The boxed figure refers to the number of the amino acid residue in the β-lactamase protein of pBR322 (J. Sutcliffe, supra). The asterisk (*) indicates that the CCT codon present at this position on pBR322 was changed to CCC as a consequence of the conversion of the PstI site in pPLa2311 to a BamHI site in pPLa8 (see above).

This configuration opens the interesting possibility of reinitiation at the HFIF AUG. Such internal reinitiation following premature termination has been observed in the repressor gene of the E. coli lactose operon (T. Platt et al. "Translational Restarts: AUG Reinitiation Of A lac Repressor Fragment" Proc. Natl. Acad. Sci. U.S.A. 69, 897-901 (1972)).

3. Construction Of Plasmid G-pPLa-HFIF-67-12:19

From the known sequence of pBR322 and the HFIF gene it can be deduced that deletion from G-pPLa-HFIF-67-12 of the small HincII fragment (from within β-lactamase up to just in front of the HFIF initiating AUG) results in a continuous translational reading frame starting at the AUG of β-lactamase and terminating at the UGA of the HFIF gene. This sequence is predicted to code for a polypeptide consisting of 82 amino acid residues from the β-lactamase gene, one amino acid coded at the fused HincII site, and followed by the complete HFIF-gene-specified polypeptide. The predicted sequence around the HincII junction point is given below.

β-lactamase moiety — GTT.AAC.AUG — HFIF coding region
Val-Asn-Met

The boxed figure refers to the number of the amino acid residue in the β-lactamase protein of pBR322 (J. Sutcliffe, supra).

G-pPLa-HFIF-67-12 DNA was partially digested with HincII. Following ligation at a DNA concentration of about 0.01 µg/ml, the DNA was cleaved with XorII, an isoschizomer of PvuI producing 3' protruding ends (R. Wang et al., Biochim. Biophys. Acta, in press), and religated at low DNA concentration. Parent G-pPLa-HFIF-67-12 contains two XorII sites: one site inactivates the kanamycin gene and the other one is located in the HincII fragment to be deleted from the plasmid. The purpose of the XorII digestion-religation step is to eliminate parent DNA molecules not cleaved by the HincII enzyme. Such molecules possess two XorII sites and under conditions used for ligation, two fragments are highly unlikely to be rejoined. Transformants were obtained in C600r$_K^-$m$_K^+$(λ) selecting for kanamycin and screened by restriction analysis for the presence of a single PvuI site. Further analysis of candidates was performed using HincII digestion. One clone missing the smallest HincII fragment but otherwise identical to G-pPLa-HFIF-67-12 was withheld and designated G-pPLa-HFIF-67-12:19. The steps used in the construction of this plasmid are shown schematically in Fig. 9. A more detailed map of this plasmid is shown in Fig. 12. The size of the plasmid (~4080 base pairs) was estimated by totaling the size of its constituent fragments, which in turn have been estimated by their relative mobility upon electrophoresis in agarose gels. E. coli K12χHI and N5219 were then transformed with the characterized plasmid G-pPLa-HFIF-67-12:19.

4. Construction Of Plasmid G-pPLc-HFIF-67-8

Plasmid G-pPLc24 offers another possibility for insertion of HFIF sequences in such a way that a fusion polypeptide can potentially be synthesized. Insertion of the BglII fragment from G-pPLa-HFIF-67-12 in the BamHI site of G-pPLc24 results in a continuous reading frame coding for 98 amino acid residues from the MS2 replicase gene (W. Fiers et al. "Complete Nucleotide Sequence Of Bacteriophage MS2 RNA: Primary And Secondary Structure Of The Replicase Gene" Nature, 260, 500-507 (1976)), 27 amino acids coded by sequences between the BglII site and the initiating AUG of HFIF, followed by the complete HFIF gene-specified polypeptide. The predicted sequence around the BamHI/BglII fusion point is shown below.

98

MS2 replicase moiety —— UGG.GAU.CTT.CAG.UTT.CGG.AGG.CAA.CCT.
Trp-Asp-Leu-Gln-Phe-Arg-Arg-Gln-Pro-

TTC.GAA.GCC.TTT.GCT.CTG.GCA.CAA.CAG.GTA.GTA.GTC.GAC.ACT.GTT.
Phe-Glu-Ala-Phe-Ala-Leu-Ala-Gln-Gln-Val-Val-Val-Asp-Thr-Val-

CGT.GTT.GTC.AAC.AUG —— HFIF coding region
Arg-Val-Val-Asn-Met

The boxed figure refers to the number of the amino acid residue in the MS2 replicase gene protein (R. Devoe et al., supra; W. Fiers et al., supra).

G-pPLa-HFIF-67-12 DNA was digested with BglII and ligated with BamHI-cleaved pPLc24 DNA. The ligation mixture was recut with BamHI to eliminate parental pPLc24 molecules and transformed into C600r$_{k}^{-}$m$_{k}^{+}$(λ) selecting for resistance to carbenicillin. Transformants were analyzed by restriction with HincII. From the known positions of restriction sites on pPLc24 one can predict that insertion of the BglII-HFIF

fragment in the sense orientation with respect to P_L should produce an extra HincII fragment of about 550 base pairs. A representative clone exhibiting this configuration was designated pPLc-HFIF-67-8. The steps used in the construction of this plasmid are shown schematically in Fig. 10. A more detailed map of this plasmid is shown in Fig. 13. The size of the plasmid (~3850 base pairs) was estimated by totaling the size of its constituent fragments, which in turn had been estimated by their relative mobility upon electrophoresis in agarose gels. E. coli K12HI and NE219 were then transformed with the characterized plasmid G-pPLc-HFIF-67-8.

ISOLATION AND CHARACTERIZATION OF HFIF MADE BY BACTERIA

A. Preparation of Bacterial Extracts

1. Induction Procedure

An aliquot from stock cultures (frozen at -80°C in 50 % glycerol - 50 % LB medium), including those of strains N100HI and M5219 transformed with any of the plasmids containing the HFIF gene as described above, was inoculated into fresh LB medium with the desired antibiotic and grown to saturation at 28°C. Two 500 ml batches of LB medium without antibiotic were inoculated with 1 ml each of saturated cells and grown with rigorous shaking to 28°C to a cell density of 2×10^8/ml. One batch was shifted to 42°C and continued to be shaken. Depending on the plasmid used the culture was harvested at various times after the shift to 42°C. The control culture remaining at 28°C was harvested at the same time as the 42°C culture. Cells were collected by centrifugation in the GSA rotor (Sorvall) at 8000 rpm for 10 minutes. The pellets were washed in 20 ml of 50 mM Tris HCl pH 7.4, 30 mM NaCl and repelleted in the SS34 rotor (Sorvall) for 10 minutes at 10,000 rpm. The pellet was quickly frozen in dry ice-methanol and stored at -80°C. When it was desired to osmotically shock the harvested cells the freezing step was omitted.

Two different procedures for lysis and extraction of the bacteria have been used.

2. Extraction Procedures

Lysis A

Cells were resuspended in a final volume of 4 ml of the above described buffer and lysozyme (Sigma) was added to 1 mg/ml. The incubation was for 30 min at 0°C. The suspension underwent two freeze-thaw cycles by sequential dipping in an ethanol-CO_2 mixture (-80°C) and a 37°C

water bath. The S-100 fraction was prepared by ultracentrifugation of the lysed bacteria (4 ml) in a Beckman 50Ti rotor for 1 hr at 60,000 rpm and 4°C, after which the supernatant was further used.

Lysis B

Lysis B was performed as described above (lysis A) except that the solution of 50 mM Tris-HCl pH 8.0, 30 mM NaCl was replaced by 50 mM Hepes (Sigma) -NaOH, pH 7.3, 30 mM NaCl, 3 mM β-mercaptoethanol and 3 % newborn calf serum (Gibco).

Osmotic Shock

Immediately after harvesting and washing, the cell-pellet was resuspended in 20 % sucrose, 100 mM EDTA, 100 mM Tris HCl pH 7.4 at a maximal cell density of .. x 10^{12}/ml. The suspension was kept on ice for 10 minutes and then centrifuged for 10 minutes at 10,000 rpm in the Sorvall SS34 rotor. The sucrose solution was carefully drained from the tube and the pellet was resuspended in an equal volume of water (cell density of 1 x 10^{10}/ml). The resuspended cells remained on ice for 10 min and were then again subjected to a centrifugation at 10,000 rpm for 10 minutes in the SS34 rotor (Sorvall). The supernatant was made 3 % in foetal calf serum, 50 mM in HEPES buffer pH 7, 30 mM in NaCl and 3 mM in β-mercaptoethanol. This supernatant is referred to as "osmotic shock supernatant". It was stored at 0°C.

3. Ammonium Sulfate Precipitation

1 ml of an $(NH_4)_2SO_4$ solution, saturated at room temperature, was added to 0.5 ml of control solution or an S-100 extract. This mixture was kept on ice for at least 30 min,

after which the precipitate was pelletted in an Eppendorf centrifuge for 10 min at room temperature. The pellet was redissolved in PBS (phosphate buffered saline).

B. Interferon Titrations

1. Direct Anti-viral assay

Human fibroblast interferon was assayed in microtiter trays (Sterilin) by a CPE (cytopathic effect)-inhibition technique in human fibroblasts trisomic for chromosome 21. The cells were seeded one day before use, incubated with serial dilutions ($\log_{10}$ = 0.5) of the sample for 24 hrs and challenged with vesicular stomatitis virus (Indiana strain), 10^{-3} dilutions of a stock containing $10^{6.9}$ mouse C-929 plaque forming units/ml. The CPE was recorded at 24 h after VSV challenge and the interferon endpoint was defined as the sample dilution causing 50% reduction of viral CPE. All assays included an internal standard of HFIF which was itself calibrated against the NIH human fibroblast reference GO23-902-527.

The cell line trisomic for chromosome 21 (henceforth referred to as T_{21}) was derived from a skin biopsy of a female patient with Down's syndrome. Its karyotype has been established and showed diploidy for all chromosomes except for chromosome 21 (trisomic). The sensitivity of this cell line to interferon appears to be comparable to the sensitivity of cell lines trisomic for chromosome 21 described by E. De Clercq et al., "Non-antiviral Activities of Interferon Are Not Controlled By Chromosome 21", Nature, 256, pp. 132-134 (1975) and E. De Clercq et al., "Chromosome 21 Does Not Code For An Interferon Receptor", Nature, 264, 249-251 (1976).

In other assays the cell line D_1SM (A. Billiau et

al., "Human Fibroblast Interferon For Clinical Trials: Production, Partial Purification And Characterization", Antimicrobial Agents And Chemotherapy, 16, 49-55 (1979)) has been used. This cell line is a diploid fibroblast disomic for chromosome 21 and derived from a two-month-old human foetus. Compared to the T_{21} cell line, F_1SH is less sensitive to HFIF by a factor of 10.

2. 2,5-A Synthetase Assay

Another method of detecting the presence of interferon is by the use of a 2,5-A synthetase assay. It has been shown that interferon induces this enzyme, which converts ATP into trimers (and to a lesser extent dimers, tetramers and multimers) of 2,5-A (S. Kimchi et al., "Kinetics Of The Induction Of Three Translation-Regulatory Enzymes By Interferon", Proc. Natl. Acad. Sci. U.S.A., 76, 3205-3212 (1979).

Confluent 25 cm^2 flasks containing cultures of F_1SH cells (A. Billiau et al., supra) were treated for 20 h with a 1:6 dilution of bacterial extracts or control interferon in MEM, 10 % fetal calf serum. The cultures were detached with trypsin (0.25 %), EDTA (0.17 %) and extensively washed with 140 mM NaCl in 35 mM Tris buffer (pH 7.5). All subsequent operations were carried out at 4°C. Cells were homogenized in 1.5-2.0 volumes of 20 mM Hepes buffer (pH 7.4) containing 10 mM KCl, 1.5 mM magnesium acetate and 0.5 mM dithiothreitol ("lysis buffer I") in a Dounce glass homogenizer. The homogenate was centrifuged for 20 min at 10,000 x g and the supernatant (S10) stored in liquid nitrogen when not used immediately.

Confluent 96-well microtiter plates (1.0^5 cells in 0.2 ml per 0.28 cm^2 well) were treated with interferon or bacterial extracts as above. After 20 h treatment, plates were cooled on ice and washed three times with 140 mM NaCl

in 35 mM Tris buffer (pH 7.5). The cultures were then lysed by adding to each well 5 µl of a solution containing 0.5 % Nonidet P.40 and 1 mM phenylmethane sulfonyl fluoride (PMSF) in lysis buffer I. After shaking vigourously for 20 min on ice the cell lysates were collected and centrifuged for 20 min at 10,000 x g as above.

3.5 µl of lysate prepared as indicated above (lysis A or lysis B) were incubated for 2 h at 31°C in 6 µl of an incubation mixture containing 100 mM potassium acetate, 25 mM magnesium acetate, 10 mM Hepes/KOH, pH 7.4, 5 mM ATP, 4 mM fructose 1,6 bis-phosphate, 1 mM dithiothreitol and 25 µg/ml poly(I)·poly(C) and 2 µCi of lyophilized (α-^{32}P)-ATP (400 Ci/mmol, from the Radiochemical Centre, Amersham, U.K.). After stopping the reaction by heating for 3 min at 95°C followed by a clarification for 2 min at 9,000 x g, the samples were treated with 150 U/ml of alkaline phosphatase from calf intestine (Boehringer, Mannheim, cat. nr. 605812) for one hour at 37°C, clarified again and spotted (1 µl per sample) on thin layer plates of polyethyleneimine-cellulose (Polygram, cel 300 PEI 20 x 20 cm from Macherey-Nagel Co., Duren, Germany). The plates were washed two times in 2 l of distilled water and dried under vacuum before chromatography in 1 M acetic acid for 2-3 h. After drying they were submitted to autoradiography for 1-24 h.

C. Detection Of MPTF Activity In Bacterial Extracts

1. Control Experiments

Two main problems resulted from the use of these extraction procedures and are important for the interpretation of the data. The bacterial extracts resulting from lysis by different procedures, as described, have been shown to contain a factor which is active in the anti-viral assay.

either the factor itself may be an anti-viral agent, or it may induce an anti-viral substance, e.g., interferon. These activities have been detected repeatedly in the S100 extracts and were often higher in extracts from E. coli HB101 than in similar extracts of the K12ΔHI or M5219 host bacteria, although this may be an effect of cell density. In control extracts of K12ΔHI or M5219, they never reached values higher than 0.7 $\log_{10}$/ml. This activity was reduced or sometimes even eliminated totally by precipitation with $(NH_4)_2SO_4$, under conditions which precipitated interferon in control experiments. Due to this contaminating activity, it is difficult to draw conclusions on the presence of trace amounts of interferon in bacterial extracts. However, it is possible to discriminate between bacterial activity and interferon activity by the use of the diploid fibroblasts E_1SM. These cells have been shown to be less sensitive to HFIF than the usual cells trisomic for chromosome 21. But the contaminating bacterial activity, in contrast to bona fide interferon, gives extremely high values on E_1SM cells. Using pMS2-7 (R. Devos et al. "Construction And Characterization Of A Plasmid Containing A Nearly Full-size DNA Copy of Bacteriophage MS2 RNA", J. Mol. Biol. 128, 595-619 (1979)) in E. coli HB101 (H. Boyer and D. Roulland-Dussoix, "A Complementation Analysis Of Restriction And Modification Of DNA In Escherichia coli", J. Mol. Biol. 41, 459-472 (1969)) or K12ΔHI-G-pPLa2311 as control lysates, data are shown in the following table, with anti-viral activity measured as $\log_{10}$ units/ml.

	T_{21}	E_1SM
HB101-pMS2-7 (lysis A)	0.7	
HB101-pMS2-7 (lysis B, but no β-mercaptoethanol and no calf serum)	<0.2	1.2
HB101-pMS2-7 (lysis B)	not done	0.7
HB101-pMS2-7 (lysis B)	0.2	1.0
HB101-pMS2-7 (lysis B)	0.7	2.5

	T_{21}	E_1SM
K12ΔHI-G-pPLa2311 (lysis B)	0.2	4.0
K12ΔHI-G-pPLa2311 (42°C; osmotic shockate)	0.5	>1.7

The presence of interferon is reflected by a different ratio of values on T_{21}:E_1SM and a high value on T_{21}. This is shown with the following data:

		T_{21}	E_1SM
osmotic shock supernatant	K12ΔHI-G-pPLa2311 (42°C)	0.5	2.5
	K12ΔHI-G-pPLa2311 (42°C) + HFIF	1.5	2.5
lysis B after $(NH_4)_2SO_4$ precipitation	HB101-pMS2-7	0.2	2.5
	HB101-pMS2-7 + HFIF (added before lysis)	2.7	2.5

For the extractions made from E. coli, either K12ΔHI or MS219 containing the expression plasmids G-pPLa-HFIF-67-12, G-pPLa-HFIF-67-12:19 or G-pPLc-HFIF-67-6, the extracts were not highly concentrated (for example, cells from 150 ml culture at 6×10^8 cells/ml were lysed and extracted in 4 ml) so that there was only a low or undetectable level of this bacterial interference.

The bacterial interference has also been shown to be detectable in the 2,5-A synthetase activity. Here it can be eliminated completely by precipitation with $(NH_4)_2SO_4$ as described above. In this way the presence of HFIF, precipitating under these conditions, can be detected in this extract. Also, extracts from HB101/G-pBR322(Pst)HFIF-6, which has an incomplete colinear coding sequence (only the last few base pairs are missing) and is thus unable to give the mature

polypeptide, has repeatedly yielded a 2,5-A synthetase activity, but so far no discernable anti-viral activity. This indicates that the 2,5-A assay cannot be regarded as the only criterion for the presence of a bacteria-made interferon. 2,5-A synthetase activity is measured by ^{32}P incorporation into the 2,5-A trimer as shown by autoradiography. Results are shown in the following table, with increasing positive values reflecting increased incorporation of ^{32}P.

extract a/pHFIF-6 → +++ ; after $(NH_4)_2SO_4$ precipitation → ++
extract b/pKS2-7 → +++ ; " → -
extract b/pKS2-7 → +++ ; " → ++
plus HFIF
} repeated 3 times

A second important problem is the low recovery of HFIF secreted by human fibroblasts during and after different experimental steps. A comparison of the recoveries of leucocyte interferon and fibroblast interferon added to an S-100 extract shows that HFIF is recovered only with 10% efficiency, in contrast to human leucocyte IF (100%) (anti-viral values are given as $\log_{10}$ units/ml; assayed on T_{21} cells).

LeIF diluted in S-100-extract of HB101-pKS2-7 (lysis A)	2.5
LeIF diluted in E-MEM plus 3% calf serum	2.7
HFIF diluted in S-100-extract of HB101-pKS2-7 (lysis A)	0.7
HFIF diluted in S-MEM-plus 3% calf serum	1.7

Other experiments where HFIF was added to the cell pellet before lysis and extraction (even with calf serum added to 3% as a stabilizer) showed that only 10-30% HFIF was recovered.

			$\log_{10}$ units/ml T_{21}	T_1 SN
		Hepes		
HB101-pMS2-7 plus HFIF	(lysis B, but no β-mercaptoethanol or calf serum)	pH 8 pH 7 pH 6	0.7(10%) 1.0(20%) 0.7(10%)	1.7 1.7 1.7
HFIF (no bacteria)	(same treatment as in lysis B)	pH 7	1.7(50%)	1.5

Further experiments were carried out to test stability and recovery of HFIF activity. Precipitation with $(NH_4)_2SO_4$ as described above, either in the presence or absence of bacterial extracts, often causes a reduction of the titer in the anti-viral assay, as shown below for samples both before and after precipitation.

	$\log_{10}$ units/ml before	after
HFIF	1.0	0.5
HFIF	2.7	2.5
HB101-pMS2-7 + HFIF (lysis B)	1.5	1.2
K12ΔHI-G-pPLa2311 (28°C) + HFIF (lysis B)	1.7	1.5
K12ΔHI-G-pPLa2311 (28°C) + HFIF (lysis B)	2.2	3.0

Dialysis of HFIF (overnight at 4°C against PBS) either in the presence or in the absence of bacterial extracts usually resulted in a decreased recovery, as shown below for samples both before and after dialysis.

	$\log_{10}$ units/ml before	after
HFIF in PBS	1.0	0.5
HFIF in PBS	2.7	2.5
K12ΔHI-G-pPLa5 (28°C) + HFIF (lysis B)	1.2	<0.2
K12ΔHI-G-pPLa2 + HFIF (lysis B)	3.0	1.7
K12ΔHI-G-pPLa3 + HFIF (lysis B)	2.2	1.0
K12ΔHI-G-pPLa2 + HFIF (lysis B)	1.5	0.5

Since HFIF is a Type I interferon its activity should be acid-stable. This was tested by dialyzing HFIF

samples in the presence or absence of bacterial extracts, overnight, in 5 mM glycine-HCl, pH 2.2, at 4°C. This treatment caused the formation of a precipitate, which was pelletted in the Eppendorf centrifuge at 12,000 x g for 2 min. The supernatant was then tested for anti-viral activity. Although some of the anti-viral activity remained following this treatment, there was a substantial loss in the amount of interferon recovered.

	$\log_{10}$ units/ml	
	before	after
MM101-pMS2-7 (lysis A) + MFIF	0.7	0.5
MM2NI-G-pPLa2311 (28°C) osmotic shockate + MFIF	1.2	1.2
MS219-G-pPLa8 (42°C) (lysis B) + MFIF	1.2	0.7
MS219-G-pPLa8 (28°C) (lysis B) + MFIF	3.0	2.0

The reductions observed with these different treatments in the control experiments must be interpreted cautiously. The lower anti-viral titers do not necessarily mean that interferon is degraded. The lowered titers may be due to non-specific sticking to dialysis membranes or to components in the bacterial extracts, e.g. membrane components. It is well established that MFIF is a hydrophobic protein (its hydrophobicity is also substantiated by its amino acid sequence) which can adhere non-specifically to tube walls or other surfaces. Bacterial MFIF, lacking glycosylation, may be even more hydrophobic. Therefore, conclusions on the recovery of the glycosylated MFIF secreted by human cells may not necessarily be extrapolated to MFIF of bacterial origin.

2. Demonstration of HFIF Activity (anti-viral activity and 2.5 A synthetase activity)

Bacterial extracts of E.coli M5219 or K12ΔHI, containing the plasmids G-pPLa-HFIF-67-12, G-pPLa-HFIF-67-12/119 or G-pPLc-HFIF-67-8, were analyzed for HFIF activity. The procedures for induction and preparation of the S-100 extracts and the osmotic-shock supernatants are as described above. 150 ml of bacterial culture (3-6 x 10^8 cells/ml) were used per experiment. All biological titers are given in $\log_{10}$ units/ml.

G-pPLa-HFIF-67-12

G-pPLa-HFIF-67-12 has been tested in the two E.coli strains M5219 and K12ΔHI and the S-100-extracts were prepared by lysis B. All samples were precipitated with $(NH_4)_2SO_4$ before testing for antiviral activity.

	T_{21}	E_1SM
K12ΔHI-G-pPLa-HFIF-67-12 (28°C)	< 0.2	< 1.0
K12ΔHI-G-pPLa-HFIF-67-12 (42°C, 90 min)	0.2/0.5	< 1.0/< 1.0
M5219-G-pPLa-HFIF-67-12 (28°C)	< 0.2	< 1.0
M5219-G-pPLa-HFIF-67-12 (42°C, 90 min)	0.7/0.7	< 1.0/< 1.2

The second figure gives the value determined on reassay of the same sample. A similar control experiment with HFIF added to the bacteria HB101-pMS2-7 before lysis of the cells indicated a recovery of 20%. It is clear that upon induction HFIF-activity can be detected in the bacterial lysate. The values, which are below the detection level in E_1SM cells, show clearly that the HFIF-activity is not due to a contaminating bacterial activity. Such a contaminating bacterial activity should in fact give at least 2.0 on E_1SM to allow values of 0.5 or 0.7 on T_{21} cells (see control experiments above).

G-pPLa-HFIF-67-12:19 and G-pPLc-HFIF-67-8

Both of these plasmids were tested in the MS219- strain and S100-extracts were prepared by lysis B. All samples were precipitated with $(NH_4)_2SO_4$ as described above and assayed for antiviral activity. The value between brackets indicates the detection level, due to some toxicity of the particular samples for the human cells in tissue culture.

i) MS219-G-pPLa-HFIF-67-12:19 (28°C)
ii) MS219-G-pPLa-HFIF-67-12:19 (42°C, 90 min, final cell density = 3×10^8/ml)
iii) MS219-G-pPLc-HFIF-67-8 (28°C)
iiii) MS219-G-pPLc-HFIF-67-8 (42°C, 120 min, final cell density = 6×10^8/ml)

	on T_{21}	on E_1SM
i)	< 0.5	2.2 (< 2.0)
ii)	2.2 (< 0.5)	2.2 (< 2.0)
iii)	< 0.5	2.2 (< 2.0)
iiii)	2.2 (< 0.5)	2.2 (< 2.0)

A control experiment with HFIF added to NB101-pMS2-7 before lysis of the cells indicated a 30% recovery. Here the high values on T_{21} cells and the ratio of activity on T_{21} over the one on E_1SM indicate that there was not an important contaminating bacterial activity (as discussed above) in the temperature induced samples.

Further evidence substantiating bacterial expression of HFIF is given by antibody neutralization experiments. The anti-interferon antiserum was produced in goats, immunized with 10^7 units of HFIF (secreted by human fibroblast cells), purified on controlled pore glass beads (A. Billiau et al., supra). After samples were analyzed for antiviral activity, serial dilu-

tions of the antiserum were added, the mixtures were incubated for 1 hr at 37°C, and applied to human diploid fibroblasts T_{21} and assayed for antiviral activity as described before. The degree of neutralization by HFIF antiserum ranges from +++ (complete neutralization) to - (no neutralization). The value between brackets indicates the approximate antiserum dilution for 50% neutralization.

1) MS219-G-pPLc-HFIF-67-8 (42°C, 180 min ; which gave 2.2 $\log_{10}$ antiviral units/ml on T_{21} cells).

2) MS219-G-pPLa-8 (42°C, 180 min) to which HFIF (from human fibroblasts) was added before lysis (which gave 1.7 $\log_{10}$ antiviral units on T_{21} cells).

dilution of antiserum	(1)	(2)
10^{-3}	+++	+++
10^{-4}	+ ($10^{-4.5}$)	+++
10^{-5}	±	+++
10^{-6}	-	± (10^{-6})
10^{-7}	-	-

HFIF has, in contrast to human leucocyte interferon the very unusual property that its activity is recovered after boiling in 1% SDS, 1 % 8-mercaptoethanol, 5 M urea (Stewart, W.E. II et al., Distinct Molecular Species Of Human Interferon, Requirements For Stabilization And Reactivation Of Human Leucocyte And Fibroblast Interferon., J. Gen. Virol., 26, 327-331, 1975), although a 100% recovery usually is not obtained. The bacterial cells of 150 ml culture were resuspended in buffer for lysis B and an equal volume of 2% SDS, 2% 8-mercaptoethanol and 10 M urea was added, followed by boiling for 2 min, after which the S-100-fraction was prepared.

a) control : MS219-G-pPLa-HFIF-67-12:19 (28°C)
b) control : 3 $\log_{10}$ units of HFIF diluted in lysis B buffer
c) MS219-G-pPLc-HFIF-67-6 (42°C, 180 min, cell density = 6×10^8/ml).

The assays were performed on T_{21}-cells. The value between brackets indicates the limit of detection, due to intrinsic toxicity.

	before dialysis	after dialysis
a)	< 1.5	< 1.5
b)	2.2 (< 1.5)	2.0 (< 0.5)
c)	3.0 (< 2.0)	2.2 (< 1.5)

The control experiment showed a recovery of about 10%. There was no detectable value in $E_1$6M in parallel control lysates. These data clearly show, that although only about 10% of added HFIF is recovered in the control experiment, an antiviral activity could clearly be detected in the temperature induced MS219-G-pPLc-HFIF-67-6-extract after this severe treatment. A higher antiviral activity was found compared to the lysis B procedure, indicating a possible adherence to cell components.

In another experiment the osmotic shock supernatant prepared as described above, was assayed on antiviral activity.

d) control : MS219-G-pPLa-HFIF-67-12:19 (28°C)
e) MS219-G-pPLc-HFIF-67-6 (28°C)
f) MS219-G-pPLc-HFIF-67-6 (42°C, 180 min, cell density = 6×10^8/ml).

The assays were performed on T_{21}-cells. The value between brackets indicates the limit of detection.

	assay before	& assay after $(NH_4)_2SO_4$-precipitation
d)	< 0.2	< 0.2
e)	< 0.2	< 0.2
f)	1.5 (< 0.2)	0.7 (< 0.2)

The recovery of HFIF was about 10% in control experiments. The values obtained with the osmotic shock supernatants show that the temperature-induced M5219-G-pPLc-HFIF-67-8-extract has an antiviral activity not present in the non-induced samples. The control lysates did not show detectable activity on E_1SM. The sample (f) after precipitation with $(NH_4)_2SO_4$ having 0.7 $\log_{10}$ units per ml was dialysed to pH 2.2 as described above and showed no decrease of activity. This acid-stability is a particular property of type I interferons e.g. HFIF.

The same bacterial samples were tested with the assay for 2'5 A synthetase, as described above, with microtiter plates, except that Hela cells were used instead of E_1SM cells. The following samples were tested :

(e) M5215-G-pPLc-HFIF-67-8 (28°C) (see above)
(f) M5219-G-pPLc-HFIF-67-8 (42°C) (see above)

The values, reflecting the 2'5 A synthetase activity, indicate the ^{32}P-radioactivity incorporated in the trimer form of 2'5 A

	(measured counts)	(after substraction of endogenous background)
1) non treated cells	3342 cpm	0 cpm
2) bacterial extract (e) : dilution 1/6	1972 cpm	-1370 cpm
3) bacterial extract (f) : dilution 1/6	6960 cpm	3618 cpm
4) bacterial extract (e) + HFIF to 1.5 $\log_{10}$ units/ml	7037 cpm	3695 cpm
5) see 3 but incubated with anti-HFIF antiserum	3950 cpm	608 cpm
6) see 4 but incubated with anti-HFIF-antiserum	2960 cpm	-382 cpm
7) control HFIF 0.5 $\log_{10}$ units/ml	4463 cpm	1120 cpm
8) control HFIF 1 $\log_{10}$ units/ml	7680 cpm	4338 cpm
9) control HFIF 1.5 $\log_{10}$ units/ml	13615 cpm	10273 cpm
10) control HFIF 2 $\log_{10}$ units/ml	25040 cpm	21698 cpm

The results on the 2'5 A synthetase activity show that the osmotic shock supernatant of the temperature induced MC219-G-pPLc-HFIF-67-8, which has antiviral activity (see above) is also inducing 2'5 A synthetase activity in contrast to the non-induced bacterial strain. This confirms the experimental data with antiviral activity. The degree of stimulation is equal to the activity of HFIF added to the control lysate (compare samples (3) and (4)). Comparison with the concentration curve (samples (7) to (10)) shows that, taking in account the dilution, an activity of $\log_{10}$ 1.7 units/ml can be estimated in both samples (3) and (4), which is compatible with the values in the direct antiviral assay i.e. 1.8 $\log_{10}$ units for both samples. Also this experiment shows that the induced activity can be neutralized by the anti-HFIF antiserum, as is the case in the antiviral assay.

The extracts (e) and (f) were also tested for antiviral activity on different cell lines of feline, monkey or rabbit origin. They did not show any detectable antiviral activity on these cells; neither did authentic HFIF, made by human cells. Also no activity was found on a feline lung cell line which was sensitive to human leucocyte interferon. These results provide further substantiation that the HFIF produced by the bacteria exhibits properties essentially identical to those of HFIF secreted by induced human fibroblast cells.

IMPROVING THE YIELD AND ACTIVITY OF POLYPEPTIDE EXHIBITING HPIF ACTIVITY PRODUCED IN ACCORDANCE WITH THIS INVENTION

The level of production of a protein is governed by three major factors: the number of copies of its gene within the cell, the efficiency with which those gene copies are transcribed and the efficiency with which they are translated. Efficiency of transcription and translation (which together comprise expression) is in turn dependent upon nucleotide sequences, normally situated ahead of the desired coding sequence. These nucleotide sequences or expression control sequences define, inter alia, the location at which RNA polymerase interacts to initiate transcription (the promoter sequence) and at which ribosomes bind and interact with the mRNA (the product of transcription) to initiate translation. Not all such expression control sequences function with equal efficiency. It is thus of advantage to separate the specific coding sequences for the desired protein from their adjacent nucleotide sequences and fuse them instead to other known expression control sequences so as to favor higher levels of expression. This having been achieved, the newly engineered DNA fragment may be inserted into a higher copy number plasmid or a bacteriophage derivative in order to increase the number of gene copies within the cell and thereby further improve the yield of expressed protein.

Several expression control sequences may be employed as described above. These include the operator, promoter and ribosome binding and interaction sequences (including sequences such as the Shine-Dalgarno sequences) of the lactose operon of E. coli ("the lac system"), the corresponding sequences of the tryptophan synthetase system

of E. coli ("the trp system"), the major operator and promoter regions of phage λ ($O_L P_L$ as described above and $O_R P_R$), a control region of filamentous single-stranded DNA phages, or other sequences which control the expression of genes of prokaryotic or eukaryotic cells and their viruses. Therefore, to improve the production of a particular polypeptide in an appropriate host, the gene coding for that polypeptide may be prepared as before and removed from a recombinant DNA molecule closer to its former expression control sequence or under the control of one of the above expression control sequences. Such methods are known in the art.

Other methods to improve the efficiency of translation involve insertion of chemically or enzymatically prepared oligonucleotides in front of the initiating codon. By this procedure a more optimal primary and secondary structure of the messenger RNA can be obtained. More specifically, the sequence can be so designed that the initiating AUG codon occurs in a readily accessible position (i.e., not masked by secondary structure) either at the top of a hairpin or in other single-stranded regions. Also the position and sequence of the aforementioned Shine-Dalgarno segment can likewise be optimized. The importance of the general structure (folding) of the messenger RNA has been documented (D. Iserentant and W. Fiers "Secondary Structure Of mRNA And Efficiency Of Translation Initiation", Gene, 9, 1-12 (1980).

Further increases in the cellular yield of the desired products depend upon an increase in the number of genes that can be utilized in the cell. This may be achieved by insertion of the RNTF gene (with or without its transcription and translation control elements) in an even higher copy number plasmid or in a temperature-controlled copy number plasmid (i.e., a plasmid which carries a mutation such that the copy number of the plasmid increases after shifting up

the temperature; B. Uhlin et al. "Plasmids With Temperature-dependent Copy Number For Amplification Of Cloned Genes And Their Products", Gene, 6, 91-106 (1979)). Alternatively, an increase in gene dosage can be achieved for example by insertion of recombinant DNA molecules engineered in the way described previously into the temperate bacteriophage λ, most simply by digestion of the plasmid with a restriction enzyme, to give a linear molecule which is then mixed with a restricted phage λ cloning vehicle (e.g., of the type described by N. E. Murray et al., "Lambdoid Phages That Simplify The Recovery Of In Vitro Recombinants", Mol. Gen. Genet. 150, 53-61 (1977) and N. E. Murray et al., "Molecular Cloning Of The DNA Ligase Gene From Bacteriophage T4", J. Mol. Biol., 132, 493-505 (1979) and the recombinant DNA molecule produced by incubation with DNA ligase. The desired recombinant phage is then selected as before and used to lysogenize a host strain of E. coli.

Particularly useful λ cloning vehicles contain a temperature-sensitive mutation in the repression gene cI and suppressible mutations in gene S, the product of which is necessary for lysis of the host cell, and gene E, the product which is the major capsid protein of the virus. With this system the lysogenic cells are grown at a relatively low temperature (e.g., 28°-32°C) and then heated to a higher temperature (e.g., 40°-45°C) to induce excision of the prophage. Prolonged growth at higher temperature leads to high levels of production of the protein, which is retained within the cells, since these are not lysed by phage gene products in the normal way, and since the phage gene insert is not encapsidated it remains available for further transcription. Artificial lysis of the cells then releases the desired product in high yield. As in this application we have also used the λ repressor system to control expression, it may

be necessary to control the excision of the prophage and hence the gene copy number by a heteroimmune control region, e.g., derived from the lambdoid phage 21.

It should be understood that polypeptides displaying HFIF activity (prepared in accordance with this invention) may be prepared in the form of a fused protein (e.g., linked to a prokaryotic N-terminal segment directing excretion), or in the form of prointerferon (e.g., starting with the interferon signal sequence which could be cleaved off upon excretion) or as mature interferon (the latter is feasible because mature fibroblast interferon starts with methionine, an amino acid used for initiation of translation). The yield of these different forms of polypeptide may be improved by any or a combination of the procedures discussed above. Also different codons for some or all of the codons used in the present DNA sequences could be substituted. These substituted codons may code for amino acids identical to those coded for by the codons replaced but result in higher yield of the polypeptide. Alternatively, the replacement of one or a combination of codons leading to amino acid replacement or to a longer or shorter HFIF-related polypeptide may alter its properties in a useful way (e.g., increase the stability, increase the solubility, increase the antiviral activity, increase the 2,5-A synthetase activity or increase the host specificity range).

Finally, the activity of the polypeptides produced by the recombinant DNA molecules of this invention may be improved by fragmenting, modifying or derivatizing the DNA sequences or polypeptides of this invention by well-known means, without departing from the scope of this invention.

Micro-organisms and recombinant DNA molecules prepared by the processes described herein are exemplified by cultures deposited in the culture collection Deutsche Sammlung von Mikroorganism in Gottingen, West Germany on April 2, 1980, and identified as HTIF-A to C:

A: E. coli MR101 (G-pBR322(Pst)/HTIF5)
B: E. coli MR101 (G-pBR322(Pst)/HTIF6)
C: E. coli MR101 (G-pBR322(Pst)/HTIF7)

These cultures were assigned accession numbers DSM 1701-1703, respectively. They are also exemplified by cultures deposited in the culture collection Deutsche Sammlung Von Mikroorganism in Gottingen, West Germany on June 5, 1980, and identified as HTIF-D to G:

D: E. coli ML219 (G-pPLa-HTIF-67-12)
E: E. coli K12MM7 (G-pPLa-HTIF-67-12)
F: E. coli MR219 (G-pPLa-HTIF-67-12A19)
G: E. coli MR219 (G-pPLa-HTIF-67-8)

These cultures were assigned accession numbers DSM 1851-1854, respectively.

While we have herein before presented a number of embodiments of this invention, it is apparent that our basic construction can be altered to provide other embodiments which utilize the processes and compositions of this invention. Therefore, it will be appreciated that the scope of this invention is to be defined by the claims appended hereto rather than the specific embodiments which have been presented herein before by way of example.

CLAIMS

1. A recombinant DNA molecule characterized by a structural gene selected from the group comprising the DNA inserts of G-pER323(Pst)/EFIF3, G-pER322(Pst)/EFIF5 or pEA322(Pst)/EFIF7, DNA sequences which hybridize to any of the foregoing DNA inserts, or DNA sequences, from whatever source obtained, including natural, synthetic, or semi-synthetic sources, related by mutation, including single or multiple, base substitutions, deletions, insertions and inversions, to any of the foregoing DNA sequences or inserts.

2. A recombinant DNA molecule characterized by a structural gene having the formula: ATGAGCGACGACTTTG
CTTCGTCGATTCTGCTCGTTGTTGCTTTCCACTACAGCTCTTTCCATGACGCTTG
AACTTCTTTGGACTCCTACGAAGAAGCAGCAATTTCAGTGTCAGAAGCTCGTTTG
CAATTGAATGGGAGCTTCAAGACTGCCTCAAGCAGAGGATGAACTTTGACACCCAT
GACGAGATTAAGCAGCTGCAGCAGTTCCAGAAGGACGACAGCTGCATTGACCATCTAT
GAAACCTTCCAGAACATCTTTGCTATTTTCAGACAAGATTCATCTACTACTCGTTTG
AATGAGCTATTGTTGAGATCTCCTCCTGGCTAATTCTCTACCAGCAGATAAACCAACTT
AAGACAGTCCTGGAAGAACTACTGGAGAAAGAAGATTTCACCAGGGGTAAACTCATG
AGCAGTCTGCACCTGAAATATATTACTGGAAGCATTCTGCATTACCTGAAGCCTAAG
GAGTACAGTCACTGTGCCTGGACCATAGTCAGAGTGGAAATCCTATGGAACTTTTAC
TTCATTAACAGACTTACAGGTTACCTCCGAAAC.

3. A recombinant DNA molecule characterized by a structural gene having the formula: ATGAGCGACAACTTG
CTTCGTCGATTCTACAAGAAGCAGCAATTTTCAGTGTCAGAAGCTTTGTGTCCAATTG
AACGGGAGCTTTGAATACTGCCTCAAGCAGAGGATGAACTTTGACACCCATGACGAG
ATTAAGCAGCTGCAGCAGTTCCAGAAGGACGACAGCTGCATTGACCATCTATTCGAG
CTCCAGAACATCTTTGCTATTTTCAGACAAGATTCATCTACTACTCGTTTGAATGAG
ACTATTGTTGAGAACCTCCTGGCTAATCTCTACCATCAGATAAACCAACTCAAGACA
GTCCTGGAAGAAAACTGGAGAAAGAAGATTTCACCAGGGGTAAACTCATGAGCAGT
CTGCACCTGAAATATATTACTGGAAGCACTCTGCATTACCTGAAGCCTAAGGAGTAC
AGTCACTGTGCCTGGACCATAGTCAGAGTGGAAATCCTATGGAACTTTTACTTCATT
AACAGACTTACAGGTTACCTCCGAAAC.

4. The recombinant DNA molecule according to claims 1 to 3, wherein the molecule comprises a cloning

vehicle having a first and a second restriction endonuclease recognition site, said structural gene being inserted between the first and second restriction sites.

5. A recombinant DNA molecule according to claims 1 to 4, selected from the group comprising G-pBR322 (Pst)/HFIF3, G-pBR322(Pst)/HFIF6 or G-pBR322(Pst)/HFIF7, molecules whose DNA inserts hybridize to the DNA inserts in any of the foregoing molecules, or molecules, from whatever source obtained, including natural, synthetic or semi-synthetic sources, related by mutation, including single or multiple, base substitutions, deletions, insertions and inversions to the DNA inserts from any of the foregoing molecules.

6. A recombinant DNA molecule characterized by a structural gene comprising a sequence of codons which codes for a polypeptide similar in amino acid sequence to those coded for by the codons of a structural gene selected from the group of genes of the formula:

ATGACCAACAAGTGTCTCCTCCAAATTGCTCTCCTGTTGTGCTTCTCCACTACAGCT
CTTTCCATGAGCTACAACTTGCTTGGATTCCTACAAAGAAGCAGCAATTTTCAGTGT
CAGAAGCTCCTGTGGCAATTGAATGGGAGGCTTGAATACTGCCTCAAGCACAGGATG
AACTTTGACATCCCTGAGGAGATTAAGCAGCTGCAGCAGTTCCAGAAGGAGGACGCC
GCATTGACCATCTATGAGATGCTCCAGAACATCTTTGCTATTTTCAGACAAGATTCA
TCTAGCACTGGCTGGAATGAGACTATTGTTGAGAACCTCCTGGCTAATGTCTATCAT
CAGATAAACCATCTGAAGACAGTCCTGGAAGAAAAACTGGAGAAAGAAGATTTCACC
AGGGGAAAACTCATGAGCAGTCTGCACCTGAAAAGATATTATGGGAGGATTCTGCAT
TACCTGAAGGCCAAGGAGTACAGTCACTGTGCCTGGACCATAGTCAGAGTGGAAATC
CTAAGGAACTTTTACTTCATTAACAGACTTACAGGTTACCTCCGAAAC, ATGAGCT
ACAACTTGCTTGGATTCCTACAAAGAAGCAGCAATTTTCAGTGTCAGAAGCTCCTGT
GGCAATTGAATGGGAGGCTTGAATACTGCCTCAAGCACAGGATGAACTTTGACATCC
CTGAGGAGATTAAGCAGCTGCAGCAGTTCCAGAAGGAGGACGCCGCATTGACCATCT
ATGAGATGCTCCAGAACATCTTTGCTATTTTCAGACAAGATTCATCTAGCACTGGCT
GGAATGAGACTATTGTTGAGAACCTCCTGGCTAATGTCTATCATCAGATAAACCATC
TGAAGACAGTCCTGGAAGAAAAACTGGAGAAAGAAGATTTCACCAGGGGAAAACTCA
TGAGCAGTCTGCACCTGAAAAGATATTATGGGAGGATTCTGCATTACCTGAAGGCCA
AGGAGTACAGTCACTGTGCCTGGACCATAGTCAGAGTGGAAATCCTAAGGAACTTTT

ACTTCATTAACAGACTTACAGGTTACCTCCGAAAC, DNA sequences which hybridize to any of the foregoing genes or DNA sequences, and DNA sequences from whatever source obtained, including natural, synthetic or semi-synthetic sources, related by mutation, including single or multiple, base substitutions, deletions, insertions and inversions, to any of the foregoing genes or sequences.

7. A host transformed with at least one recombinant DNA molecule according to any of the preceding claims.

8. The transformed host of claim 7 characterized in that the host is selected from the group comprising strains of E. coli, Pseudomonas, Bacillus subtilis, Bacillus stearothermophilus, other bacilli, yeasts, other fungi, animal and plant hosts or human tissue cells.

9. The transformed host according to claims 7 to 8, characterized in that it comprises E. coli HB101 (G-pBR322(Pst)/HFIF3), E. coli HB101 (G-pBR322(Pst)/HFIF6), or E. coli HB101 (G-pBR322(Pst)/HFIF7).

10. A gene selected from the group comprising the DNA inserts of G-pBR322(Pst)/HFIF3, G-pBR322(Pst)/HFIF6 or G-pBR322(Pst)/HFIF7, DNA sequences which hybridize to any of the foregoing DNA inserts, or DNA sequences, from whatever source obtained, including natural, synthetic or semi-synthetic sources, related by mutation, including single or multiple, base substitutions, deletions, insertions and inversions to any of the foregoing DNA sequences or inserts.

11. A gene selected from the group of genes of the formula: ATGACCAACAAGTGTCTCCTCCAAATTGCTCTCCTGTTGT
GCTTCTCCACTACAGCTCTTTCCATGAGCTACAACTTGCTTGGATTCCTACAAAGAA
GCAGCAATTTTCAGTGTCAGAAGCTCCTGTGGCAATTGAATGCGATGCTTGAATACT
GCCTCAAGCACAGGATGAACTTTGACATCCCTGAGGAGATTAAGCAGCTGCAGCAGT
TCCAGAAGGAGGACGCCGCATTGACCATCTATGAGATGCTTCAGAACATCTTTGCTA
TTTTCAGACAAGATTCATCTAGCACTGCCTGGAATGAGACTATTGTTGAGAACCTCC

TGGCTAATGTCTATCATCAGATAAACCATCTGAAGACAGTCCTGGAAGAAAAACTGG
AGAAAGAAGATTTCACCAGGGGAAAACTCATGAGCAGTCTGCACCTGAAAAGATATT
ATGGGAGGATTCTGCATTACCTGAAGGCCAAGGAGTACAGTCACTGTGCCTGGACCA
TAGTCAGAGTGGAAATCCTAAGGAACTTTTACTTCATTAACAGACTTACAGGTTACC
TCCGAAAC, ATGAGCTACAACTTGCTTGGATTCCTACAAAGAAGCAGCAATTTTCA
GTGTCAGAAGCTCCTGTGGCAATTGAATGGGAGGCTTGAATACTGCCTCAAGCACAG
GATGAACTTTGACATCCCTGAGGAGATTAAGCAGCTGCAGCAGTTCCAGAAGGAGGA
CGCCGCATTGACCATCTATGAGATGCTCCAGAACATCTTTGCTATTTTCAGACAAGA
TTCATCTAGCACTGGCTGGAATGAGACTATTGTTGAGAACCTCCTGGCTAATGTCTA
TCATCAGATAAACCATCTGAAGACAGTCCTGGAAGAAAAACTGGAGAAAGAAGATTT
CACCAGGGGAAAACTCATGAGCAGTCTGCACCTGAAAAGATATTATGGGAGGATTCT
GCATTACCTGAAGGCCAAGGAGTACAGTCACTGTGCCTGGACCATAGTCAGAGTGGA
AATCCTAAGGAACTTTTACTTCATTAACAGACTTACAGGTTACCTCCGAAAC, DNA sequences which hybridize to any of the foregoing genes, DNA sequences, from whatever source obtained, including natural, synthetic or semi-synthetic sources, related by mutation, including single or multiple, base substitutions, deletions, insertions and inversions to any of the foregoing genes or DNA sequences, or genes comprising a sequence of codons which codes for a polypeptide similar in amino acid sequence to those coded for by any of the foregoing DNA sequences or genes.

12. A screening process for DNA sequences characterized by the step of determining whether said DNA sequence hybridizes to at least one of the DNA inserts of G-pBR322(Pst)/HFIF3, G-pBR322(Pst)/HFIF6 or G-pBR322(Pst)/HFIF7, DNA sequences which hybridize to any of the foregoing DNA inserts and DNA sequences, from whatever source obtained, including natural, synthetic or semi-synthetic sources, related by mutation, including single or multiple, base substitutions, deletions, inversions and insertions to any of the foregoing DNA inserts or sequences.

13. A screening process for DNA sequences characterized by the step of determining whether said DNA sequences hybridizes to at least one of a gene selected from the group of ATGACCAACAAGTGTCTCCTCCAAATTGCTCTCCTGTTGTGCTT

CTCCACTACAGCTCTTTCCATGAGCTACAACTTGCTTGGATTCCTACAAAGAAGCAG
CAATTTTCAGTGTCAGAAGCTCCTGTGGCAATTGAATGGGAGGCTTGAATACTGCCT
CAAGCACAGGATGAACTTTGACATCCCTGAGGAGATTAAGCAGCTGCAGCAGTTCCA
GAAGGAGGACGCCCCATTGACCATCTATGAGATGCTCCAGAACATCTTTGCTATTTT
CAGACAAGATTCATCTAGCACTGGCTGGAATGAGACTATTGTTGAGAACCTCCTGGC
TAATGTCTATCATCAGATAAACCATCTGAAGACAGTCCTGGAAGAAAAACTGGAGAA
AGAAGATTTCACCAGGGGAAAACTCATGAGCAGTCTGCACCTGAAAAGATATTATGG
GAGGATTCTGCATTACCTGAAGGCCAAGGAGTACAGTCACTGTGCCTGGACCATAGT
CAGAGTGGAAATCCTAAGGAACTTTTACTTCATTAACAGACTTACAGGTTACCTCCG
AAAC, ATGAGCTACAACTTGCTTGGATTCCTACAAAGAAGCAGCAATTTTCAGTGT
CAGAAGCTCCTGTGGCAATTGAATGGGAGGCTTGAATACTGCCTCAAGCACAGGATG
AACTTTGACATCCCTGAGGAGATTAAGCAGCTGCAGCAGTTCCAGAAGGAGGACGCC
GCATTGACCATCTATGAGATGCTCCAGAACATCTTTGCTATTTTCAGACAAGATTCA
TCTAGCACTGGCTGGAATGAGACTATTGTTGAGAACCTCCTGGCTAATGTCTATCAT
CAGATAAACCATCTGAAGACAGTCCTGGAAGAAAAACTGGAGAAAGAAGATTTCACC
AGGGGAAAACTCATGAGCAGTCTGCACCTGAAAAGATATTATGGGAGGATTCTGCAT
TACCTGAAGGCCAAGGAGTACAGTCACTGTGCCTGGACCATAGTCAGAGTGGAAATC
CTAAGGAACTTTTACTTCATTAACAGACTTACAGGTTACCTCCGAAAC, DNA
sequences which hybridize to any of the foregoing genes,
DNA sequences, from whatever source obtained including
natural, synthetic or semi-synthetic sources related by
mutation, including single or multiple, base substitutions,
deletions, insertions and inversions to any of the
foregoing genes or DNA sequences.

14. The process of any of claims 12 to 13 characterized in that the DNA sequence screened is selected from the group comprising DNA sequences from natural sources, synthetic DNA sequences, DNA sequences from recombinant DNA molecules or DNA sequences, which are a combination of the foregoing.

15. A method for producing a DNA sequence comprising the steps of preparing a recombinant DNA molecule characterized by an inserted structural gene, said gene being selected from the group comprising the DNA inserts of G-pBR322 (Pst)/HFIF3, G-pBR322(Pst)/HFIF6 or G-pBR322 (Pst)/HFIF7, DNA sequences which hybridize

to any of the foregoing DNA inserts, or DNA sequences, from whatever source obtained, including natural, synthetic or semi-synthetic sources related by mutation, including single or multiple, base substitutions, deletions, insertions and inversions, to any of the foregoing DNA sequences or inserts, or DNA inserts which comprise a sequence of codons which code for a polypeptide similar in amino acid sequence to those polypeptides coded for by any of the foregoing DNA inserts or sequences; transforming an appropriate host with said recombinant DNA molecule; culturing said host; and separating said DNA sequences.

16. A method for producing a DNA sequence comprising the steps of culturing a host transformed with at least one recombinant DNA molecule selected from the group comprising G-pBR322(Pst)/HFIF3, G-pBR322(Pst)/HFIF6, or G-pBR322(Pst)/HFIF7, molecules whose DNA inserts hybridize to the DNA inserts of any of the foregoing molecules, molecules whose DNA inserts, from whatever source obtained including natural, synthetic or semi-synthetic sources, are related by mutation, including single or multiple, base substitutions, deletions, insertions and inversions to the DNA inserts of any of the foregoing molecules, or molecules whose DNA inserts comprise a sequence of codons which code for a polypeptide similar in amino acid sequence to those polypeptides coded for by the DNA inserts of any of the foregoing molecules.

17. A method for producing a DNA sequence comprising the steps of preparing a recombinant DNA molecule characterized by an inserted structural gene, said gene being selected from the group comprising

ATGACCAACAAGTGTCTCCTCCAAATTGCTCTCCTGTTGTGCCTTCTCCACTACAG
CTCTTTCCATGAGCTACAACTTGCTTGGATTCCTACAAAGAAGCAGCAAATTTTCA
GTGTCAGAAGCTCCTGTGGCAATTGAATGGGAGGCTTGAATACTGCCTCAAGCAC
ACGATGAACTTTGACATCCCTGAGGAGATTAAGCAGCTGCAGCAGTTCCAGAAGG

AGGACGCCGCATTGACCATCTATGAGATGCTCCAGAACATCTTTGCTATTTTCAG
ACAAGATTCATCTAGCACTGGCTGGAATGAGACTATTGTTGAGAACCTCCTGGCT
AATGTCTATCATCAGATAAACCATCTGAAGACAGTCCTGGAAGAAAAACTGGAGA
AAGAAGATTTCACCAGGGGAAAACTCATGAGCAGTCTGCACCTGAAAAGATATTAT
GGGAGGATTCTGCATTACCTGAAGGCCAAGGAGTACAGTCACTGTGCCTGGACCAT
AGTCAGAGTGGAAATCCTAAGGAACTTTTACTTCATTAACAGACTTACAGGTTACC
TCCGAAAC. ATGAGCTACAACTTGCTTGGATTCCTACAAAGAAGCAGCAATTTTC
AGTGTCAGAAGCTCCTCTGGCAATTGAATGGGAGGCTTGAATACTGCCTCAAGCAC
AGGATGAACTTTGACATCCCTGAGGAGATTAAGCAGCTGCAGCAGTTCCAGAAGGA
GGACGCCGCATTGACCATCTATGAGATGCTCCAGAACATCTTTGCTATTTTCAGAC
AAGATTCATCTAGCACTGGCTGGAATGAGACTATTGTTGAGAACCTCCTGGCTAAT
GTCTATCATCAGATAAACCATCTGAAGACAGTCCTGGAAGAAAAACTGGAGAAAGA
AGATTTCACCAGGGGAAAACTCATGAGCAGTCTGCACCTGAAAAGATATTATGGGA
GGATTCTGCATTACCTGAAGGCCAAGGAGTACAGTCACTGTGCCTGGACCATAGTC
AGAGTGGAAATCCTAAGGAACTTTTACTTCATTAACAGACTTACAGGTTACCTCC
GAAAC. DNA sequences which hybridize to any of the foregoing genes, DNA sequences, from whatever source obtained, including natural, synthetic or semi-synthetic sources, related by mutation, including single or multple, base substitutions, deletions, insertions and inversions to any of the foregoing genes or DNA sequences, or DNA sequences comprising a sequence of codons which codes for a polypeptide similar in amino acid sequence to those polypeptides coded for by the codons of any of the foregoing genes or DNA sequences; transforming an appropriate host with said recombinant DNA molecule; culturing said host and separating said DNA sequence.

18. A method for producing a DNA sequence comprising the step of culturing a host transformed with at least one recombinant DNA molecule selected from the group comprising molecules whose DNA inserts are selected from the group of genes comprising ATGACCAACAAGTGTCTCCT
CCAAATTGCTCTCCTGTTGTGCTTCTCCACTACAGCTCTTTCCATGAGCTACAAC
TTGCTTGGATTCCTACAAAGAAGCAGCAATTTTCAGTGTCAGAAGCTCCTGTGGC
AATTGAATGGGAGGCTTGAATACTGCCTCAAGCACAGGATGAACTTTGACATCCC
TGAGGAGATTAAGCAGCTGCAGCAGTTCCAGAAGGAGGACGCCGCATTGACCATC

TATGAGATGCTCCAGAACATCTTTGCTATTTTCAGACAAGATTCATCTAGCACTG
GCTGGAATGAGACTATTGTTGAGAACCTCCTGGCTAATGTCTATCATCAGATAAA
CCATCTGAAGACAGTCCTGGAAGAAAAACTGGAGAAAGAAGATTTCACCAGGGGA
AAACTCATGAGCAGTCTGCACCTGAAAAGATATTATGGGAGGATTCTGCATTACC
TGAAGGCCAAGGAGTACAGTCACTGTGCCTGGACCATAGTCAGAGTGGAAATCCT
AAGGAACTTTTACTTCATTAACAGACTTACAGGTTACCTCCGAAAC, ATGAGCT
ACAACTTGCTTGGATTCCTACAAAGAAGCAGCAATTTTCAGTGTCAGAAGCTCCT
GTGGCAATTGAATGGGAGGCTTGAATACTGCCTCAAGCACAGGATGAACTTTGAC
ATCCCTGAGGAGATTAAGCAGCTGCAGCAGTTCCAGAAGGAGGACGCCGCATTGA
CCATCTATGAGATGCTCCAGAACATCTTTGCTATTTTCAGACAAGATTCATCTAG
CACTGGCTGGAATGAGACTATTGTTGAGAACCTCCTGGCTAATGTCTATCATCAG
ATAAACCATCTGAAGACAGTCCTGGAAGAAAAACTGGAGAAAGAAGATTTCACCA
GGGGAAAACTCATGAGCAGTCTGCACCTGAAAAGATATTATGGGAGGATTCTGCA
TTACCTGAAGGCCAAGGAGTACAGTCACTGTGCCTGGACCATAGTCAGAGTGGAA
ATCCTAAGGAACTTTTACTTCATTAACAGACTTACAGGTTACCTCCGAAAC, DNA sequences which hybridize to any of the foregoing genes, DNA sequences, from whatever source obtained, including natural, synthetic or semi-synthetic sources, related by mutation, including single or multiple, base substitutions, deletions, insertions and inversions to any of the foregoing genes or DNA sequences, or DNA sequences which code for a polypeptide similar in amino acid sequence to those polypeptides coded for by any of the foregoing genes or DNA sequences.

19. The method of any of claims 15 to 18 characterized in that the host is selected from the group comprising strains of E. coli, Pseudomonas, Bacillus subtilis, Bacillus stearothermophilus, other bacilli, yeasts, other fungi, animal and plant hosts, or human tissue cells.

20. A recombinant DNA molecule characterized by a structural gene coding for a polypeptide displaying an immunological or biological activity of human fibroblast interferon.

21. A recombinant DNA molecule characterized by a structural gene selected from the group comprising

the DNA inserts of G-pPLa-HFIF-67-12, G-pPLa-HFIF-67-12△19, G-pPLc-HFIF-67-8, DNA sequences which hybridize to any of the foregoing DNA inserts or DNA sequences, from whatever source obtained, including natural, synthetic, or semi-synthetic sources, related by mutation, including single or multiple, base substitutions, deletions, insertions and inversions, to any of the foregoing DNA sequences or inserts.

22. A recombinant DNA molecule according to claims 20 to 21, characterized in that structural gene is operatively linked to an expression control sequence.

23. A recombinant DNA molecule according to claims 20 to 22, wherein the molecule comprises a cloning vehicle having a first and a second restriction endonuclease recognition site, said structural gene being inserted between the first and second restriction sites.

24. A recombinant DNA molecule according to claim 23, characterized in that the expression control sequence is also inserted into the cloning vehicle.

25. A recombinant DNA molecule according to claims 22-24, characterized in that the expression control sequence is selected from the group comprising the E. coli lac system, the E. coli trp system, major operator and promoter regions of phage λ, the control region of Filamenteous single stranded DNA phages, the β-lactamase system of E. coli plasmids or other sequences which control the expression of genes of prokaryotic or eukaryotic cells and their viruses.

26. A recombinant DNA molecule selected from the group comprising G-pPLa-HFIF-67-12, G-pPLa-HFIF-67-12△19 or G-PLc-HFIF-67-8.

27. A host transformed with at least one recombinant DNA molecule according to any of claims 20 to 26.

28. A transformed host of claim 27 characterized in that the host is selected from the group comprising

strains of E. coli, Pseudomonas, Bacillus subtilis, Bacillus steerothermophilus, other bacilli, yeasts, other fungi, animal and plant hosts or human tissue cells.

29. A transformed host of claims 27 to 28 characterized in that it comprises a transformed host selected from the group comprising E. coli MS219 (G-pPLa-HFIF-67-12), E. coli K12△HI (G-pPLa-HFIF-67-12), E. coli MS219 (G-pPLa-HFIF-67-12△19), or E. coli MS219 (G-pPLc-HFIF-67-8).

30. A DNA sequence coding for a polypeptide displaying an immunological or biological activity of human fibroblast interferon, said sequence producing in a recombinant DNA molecule transformed host said polypeptide.

31. A DNA sequence of claim 30 selected from the group comprising the DNA inserts of G-pPLa-HFIF-67-12, G-pPLA-HFIF-67-12△19, G-pPLc-HFIF-67-8, DNA sequences which hybridize to any of the foregoing DNA inserts, DNA sequences, from whatever source obtained, including natural, synthetic or semi-synthetic sources, related by mutation, including single or multiple, base substitutions, deletions, insertions, and inversions to any of the foregoing DNA inserts, or DNA sequences comprising sequences of codons which on expression code for polypeptides similar in immunological or biological activity to those coded for by any of the foregoing DNA sequences or inserts.

32. A polypeptide or fragments and derivatives thereof displaying an immunological or biological activity of human fibroblast interferon produced by a host transformed with a recombinant DNA molecule according to any one of claims 20-26.

33. A polypeptide of claim 32 characterized in that the structural gene which codes therefor is

selected from the group comprising the DNA inserts of G-pFLa-HFIF-67-12, G-pFLa-HFIF-67-12Δ19, G-pFLc-HFIF-67-8, DNA sequences which hybridize to any of the foregoing DNA inserts, DNA sequences, from whatever source obtained, including natural, synthetic or semi-synthetic sources, related by mutation, including single or multiple, base substitutions, deletions, insertions and inversions to any of the foregoing DNA sequences or inserts or DNA sequences which on expression code for a polypeptide similar in immunological or biological activity to a polypeptide coded for on expression of any of the foregoing DNA sequences or inserts.

34. A polypeptide or fragments and derivatives thereof selected from the group comprising polypeptides of the formula: Met-Thr-Asn-Lys-Cys-Leu-Leu-Gln-Ile-Ala-Leu-Leu-Leu-Cys-Phe-Ser-Thr-Thr-Ala-Leu-Ser-Met-Ser-Tyr-Asn-Leu-Leu-Gly-Phe-Leu-Gln-Arg-Ser-Ser-Asn-Phe-Gln-Cys-Gln-Lys-Leu-Leu-Trp-Gln-Leu-Asn-Gly-Arg-Leu-Glu-Tyr-Cys-Leu-Lys-Asp-Arg-Met-Asn-Phe-Asp-Ile-Pro-Glu-Glu-Ile-Lys-Gln-Leu-Gln-Gln-Phe-Gln-Lys-Glu-Asp-Ala-Ala-Leu-Thr-Ile-Tyr-Glu-Met-Leu-Gln-Asn-Ile-Phe-Ala-Ile-Phe-Arg-Gln-Asp-Ser-Ser-Ser-Thr-Gly-Trp-Asn-Glu-Thr-Ile-Val-Glu-Asn-Leu-Leu-Ala-Asn-Val-Tyr-His-Gln-Ile-Asn-His-Leu-Lys-Thr-Val-Leu-Glu-Glu-Lys-Leu-Glu-Lys-Glu-Asp-Phe-Thr-Arg-Gly-Lys-Leu-Met-Ser-Ser-Leu-His-Leu-Lys-Arg-Tyr-Tyr-Gly-Arg-Ile-Leu-His-Tyr-Leu-Lys-Ala-Lys-Glu-Tyr-Ser-His-Cys-Ala-Trp-Thr-Ile-Val-Arg-Val-Glu-Ile-Leu-Arg-Asn-Phe-Tyr-Phe-Ile-Asn-Arg-Leu-Thr-Gly-Tyr-Leu-Arg-Asn, polypeptides of the formula: Met-Ser-Tyr-Asn-Leu-Leu-Gly-Phe-Leu-Gln-Arg-Ser-Ser-Asn-Phe-Gln-Cys-Gln-Lys-Leu-Leu-Trp-Gln-Leu-Asn-Gly-Arg-Leu-Glu-Tyr-Cys-Leu-Lys-Asp-Arg-Met-Asn-Phe-Asp-Ile-Pro-Glu-Glu-Ile-Lys-Gln-Leu-Gln-Gln-Phe-Gln-Lys-Glu-Asp-Ala-Ala-Leu-Thr-Ile-Tyr-Glu-Met-Leu-Gln-Asn-Ile-Phe-Ala-Ile-Phe-Arg-Gln-Asp-Ser-Ser-Ser-Thr-Gly-Trp-Asn-Glu-Thr-Ile-Val-Glu-Asn-Leu-Leu-Ala-Asn-Val-Tyr-His-Gln-Ile-Asn-His-Leu-Lys-Thr-Val-Leu-Glu-Glu-Lys-Leu-Glu-Lys-Glu-

Asp-Phe-Thr-Arg-Gly-Lys-Leu-Met-Ser-Ser-Leu-His-Leu-Lys-Arg-Tyr-Tyr-Gly-Arg-Ile-Leu-His-Tyr-Leu-Lys-Ala-Lys-Glu-Tyr-Ser-His-Cys-Ala-Trp-Thr-Ile-Val-Arg-Val-Glu-Ile-Leu-Arg-Asn-Phe-Tyr-Phe-Ile-Asn-Arg-Leu-Thr-Gly-Tyr-Leu-Arg-Asn, or polypeptides from whatever source obtained related to any of the foregoing polypeptides by mutation, including single or multiple, base substitutions, deletions, insertions and inversions, to any of the DNA sequences which code for them.

35. A composition for rendering humans resistant to virus strains comprising at least one polypeptide produced by a host transformed with a recombinant DNA molecule according to claims 20 to 26.

36. A composition for rendering humans resistant to virus strains comprising at least one polypeptide according to claims 32 to 34.

37. A composition for treating human cancers comprising at least one polypeptide produced by a host transformed with a recombinant DNA molecule according to claims 20 to 26.

38. A composition for treating human cancers comprising at least one polypeptide according to claims 32 to 34.

39. A method for producing a polypeptide comprising the steps of preparing a recombinant DNA molecule characterized by an inserted structural gene, said gene being selected from the group comprising the DNA inserts of G-pPLa-HFIF-67-12, G-pPLa-HFIF-67-12Δ19, G-pPLc-HFIF-67-8, DNA sequences which hybridize to any of the foregoing DNA inserts, or DNA sequences, from whatever source obtained, including natural, synthetic or semi-synthetic sources related by mutation, including single or multiple, base substitutions, deletions, insertions and inversions, to any of the foregoing DNA sequences or inserts and having operatively linked thereto an expression control sequence;

transforming an appropriate host with said recombinant DNA molecule; culturing said host; and collecting said polypeptide.

40. A method for producing a polypeptide comprising the steps of culturing a host transformed with at least one recombinant DNA molecule selected from the group comprising G-pPLa-HFIF-67-12, G-pPLa-HFIF-67-12Δ19, G-pPLc-HFIF-67-8, molecules whose DNA inserts hybridize to the DNA inserts of any of the foregoing molecules, molecules whose DNA inserts, from whatever source obtained, including natural, synthetic or semi-synthetic sources are related by mutation, including single or multiple, base substitutions, deletions, insertions and inversions to the DNA inserts of any of the foregoing molecules, or molecules whose DNA inserts comprise a sequence of codons which on expression code for a polypeptide similar in immunological or biological activity to a polypeptide coded for on expression of the DNA inserts of any of the foregoing molecules and collecting said polypeptide.

41. A method of rendering human resistant to virus strains comprising the steps of treating humans in a pharmaceutically acceptable manner with a composition comprising at least one polypeptide produced by a host transformed with a recombinant DNA molecule according to claims 20 to 26.

42. A method of rendering human resistant to virus strains comprising the steps of treating humans in a pharmaceutically acceptable manner with a composition comprising at least one polypeptide according to claims 32 to 34.

43. A method for treating human cancers or tumors comprising the steps of treating humans in a pharmaceutically acceptable manner with a composition comprising at least one polypeptide produced by a host transformed with a recombinant DNA molecule according to claims 20 to 26.

44. A method for treating human cancers or tumors comprising the steps of treating humans in a pharmaceutically acceptable manner with a composition comprising at least one polypeptide according to claims 32 to 34.

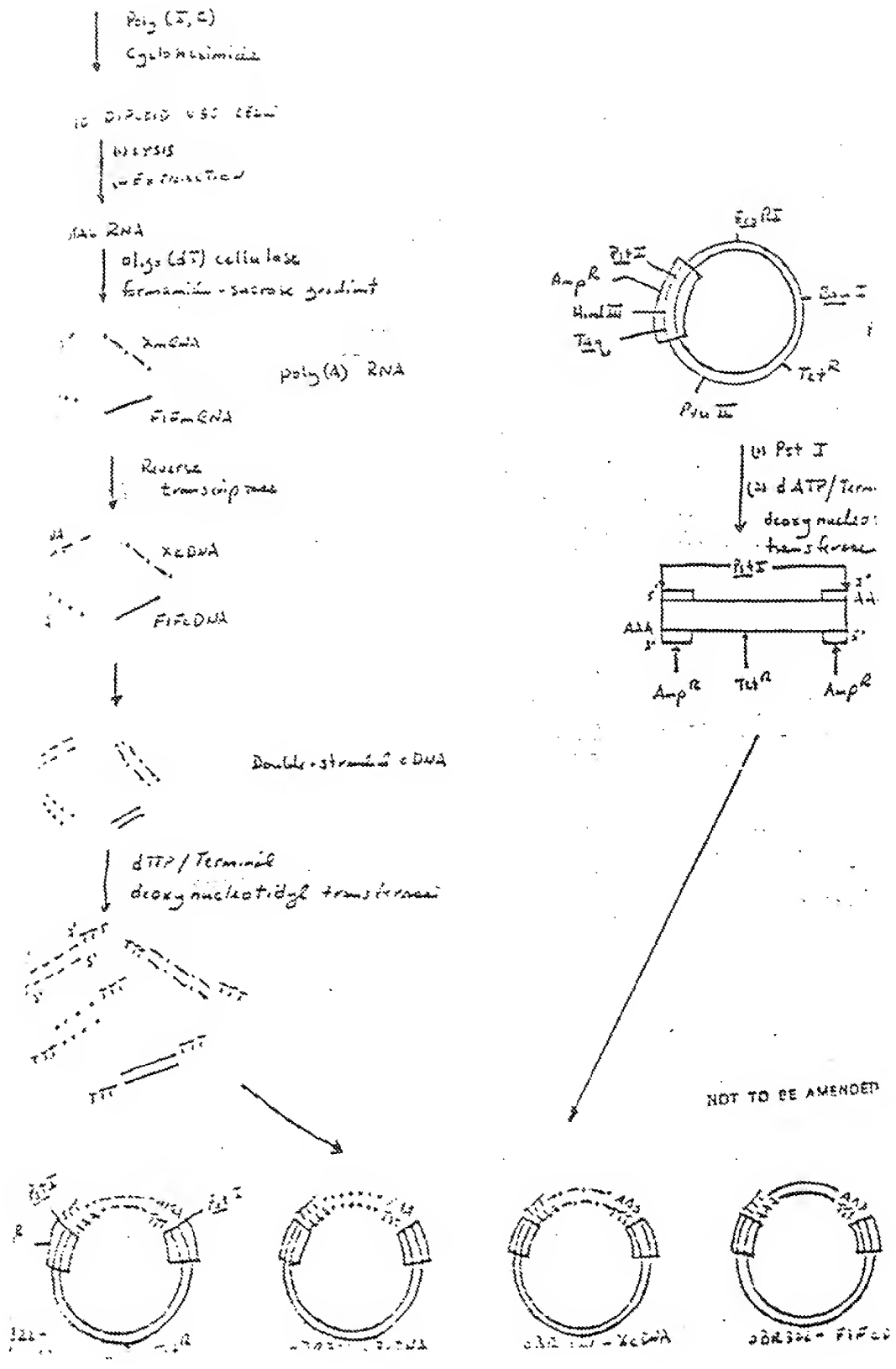
45. The method of claim 39 wherein the step of collecting the polypeptide includes a step selected from the group comprising osmotic shockate, boiling in SDS, urea and β-mercaptoethanol, boiling in basic solution or other similarly harsh treatments.

46. The method of claim 40 wherein the step of collecting the polypeptide includes a step selected from the group comprising osmotic shockate, boiling in SDS, urea and β-mercaptoethanol, boiling in basic solution or other similarly harsh treatments.

47. A human fibroblast interferon like polypeptide selected from the group comprising HFIF-67-12, HFIF-67-12Δ19, or HFIF-67-8.

ABSTRACT

Recombinant DNA molecules and hosts transformed with them which produce polypeptides displaying a biological or immunological activity of human fibroblast interferon, the genes coding for these polypeptides and methods of making and using these molecules, hosts, genes and polypeptides. The recombinant DNA molecules are characterized by structural genes that code for a polypeptide displaying a biological or immunological activity of human fibroblast interferon. In appropriate hosts these molecules permit the production and identification of genes and polypeptides displaying a biological or immunological activity of human fibroblast interferon and their use in antiviral and antitumor or anticancer agents.


Poly (A) RNA
Reverse transcriptase
Double-stranded cDNA
dTTP / Terminal deoxynucleotidyl transferase
Pst I
dATP / Terminal deoxynucleotidyl transferase
Amp^R
Tet^R
NOT TO BE AMENDED

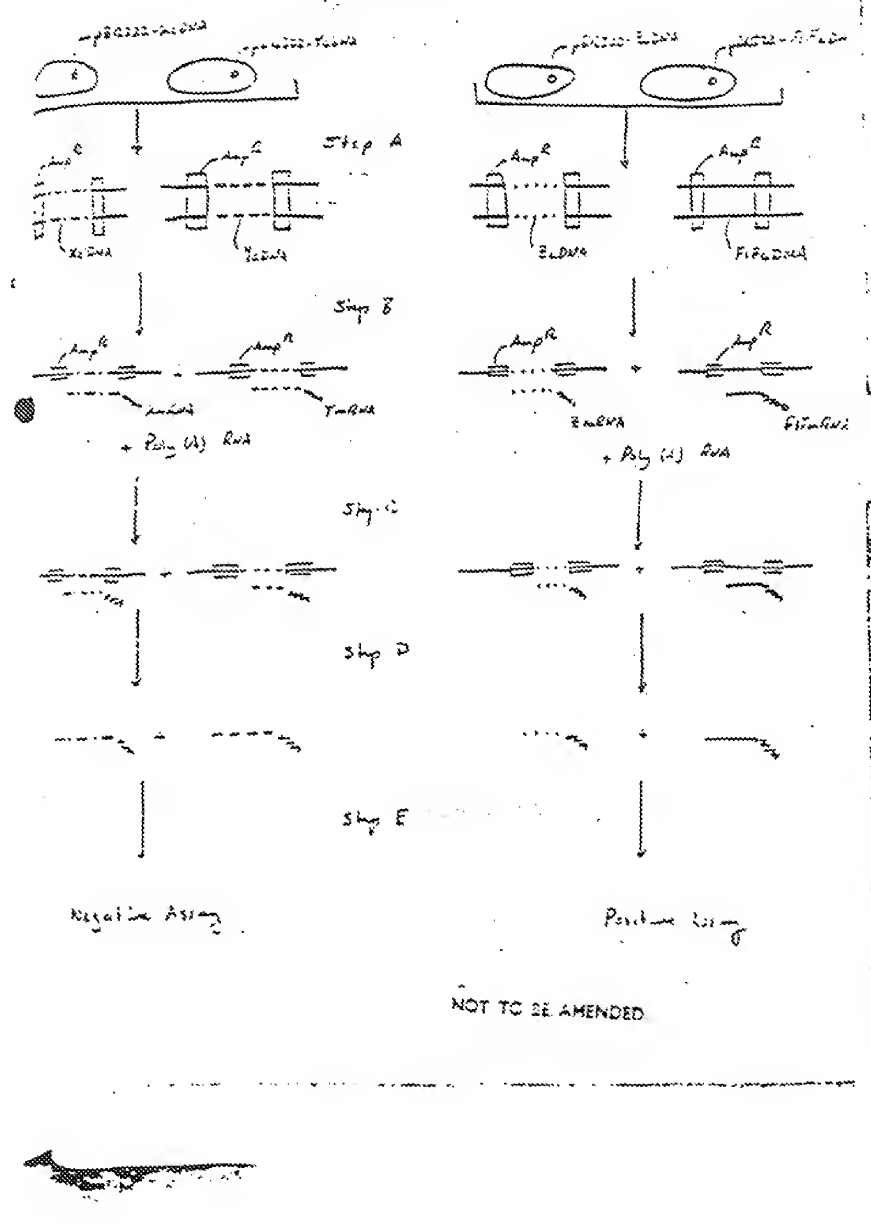


FIG. 3

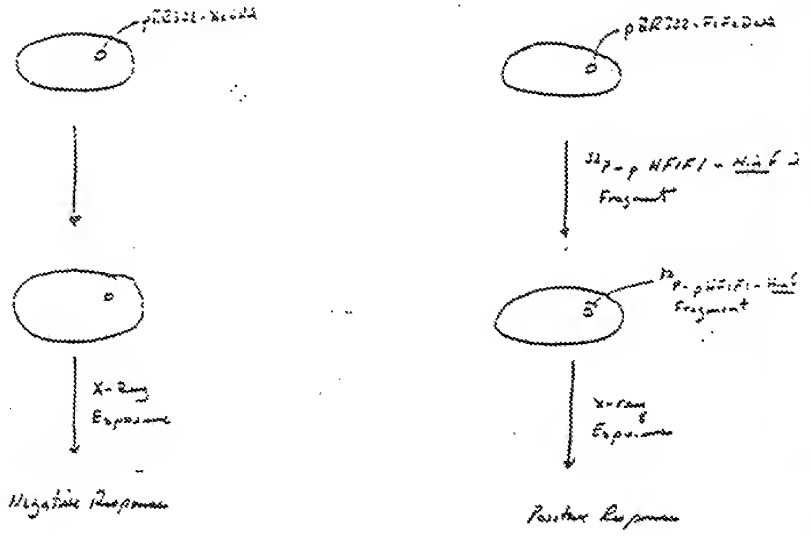


FIG. 4

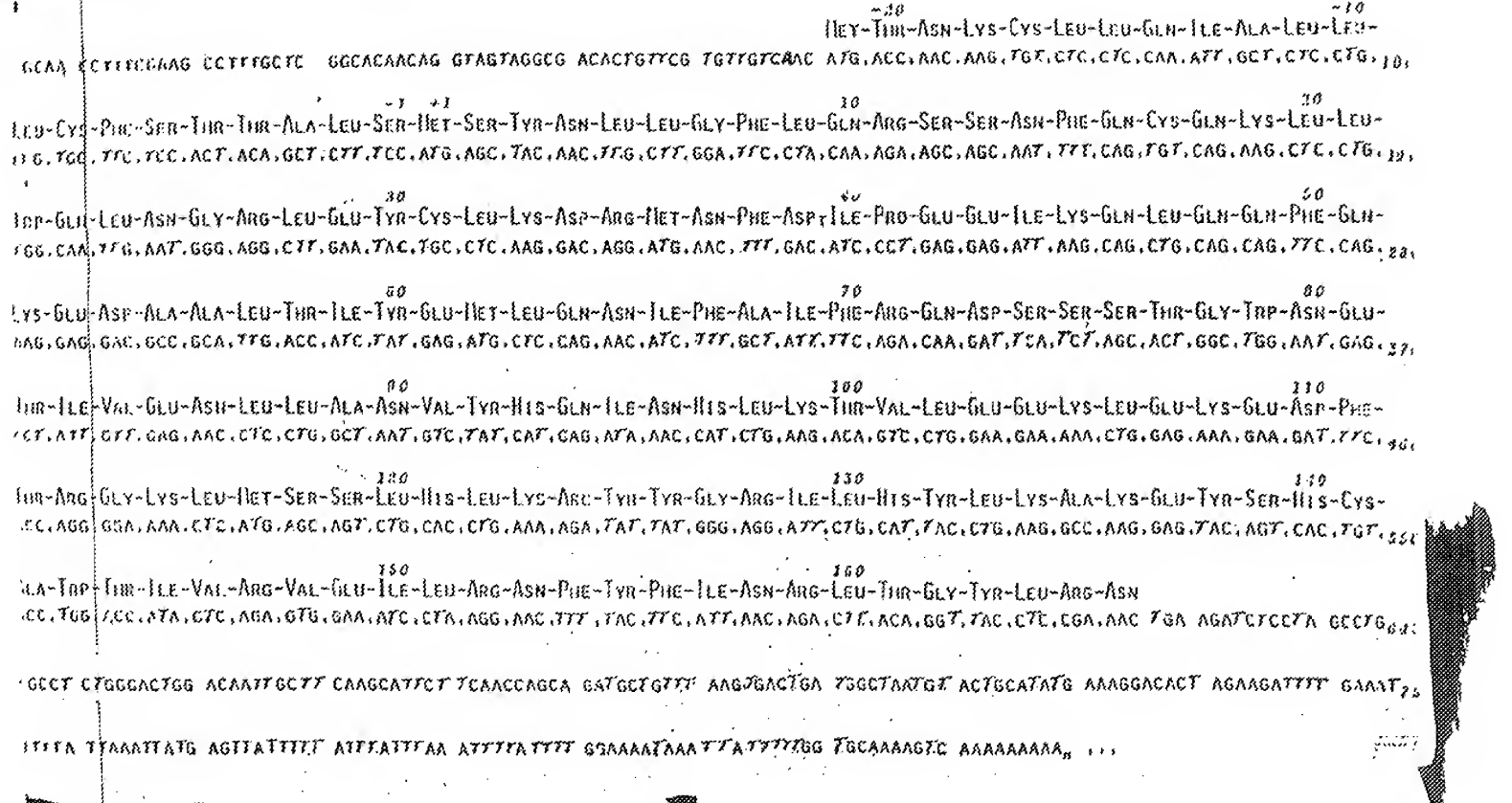


FIG. 5

Amino Acid Composition of Human Fibroblast Interferon

Amino Acid	Composition from direct analysis by Tan et al.	from direct analysis by Knight et al.	deduced from nucleotide sequence	
Asp	20.6	18.9	5	17
Asn			12	
Thr	8.0	6.8	7	
Ser	11.7	10.5	9	
Glu	27.5	27.0	13	24
Gln			11	
Pro	4.4	2.7	1	
Gly	5.4	7.8	6	
Ala	9.3	10.0	6	
Cys	N.D.	1.7	3	
Val	7.9	6.0	5	
Met	trace	2.9	4	
Ile	10.0	9.0	11	
Leu	26.9	20.4	24	
Tyr	3.2	7.5	10	
Phe	7.7	5.4	9	
His	4.6	4.9	5	
Lys	12.3	11.6	11	
Arg	8.6	10.9	11	
Trp	0.0	1.0	3	
TOTAL	168	169	168	

Fig. 6

NOT TO BE AMENDED

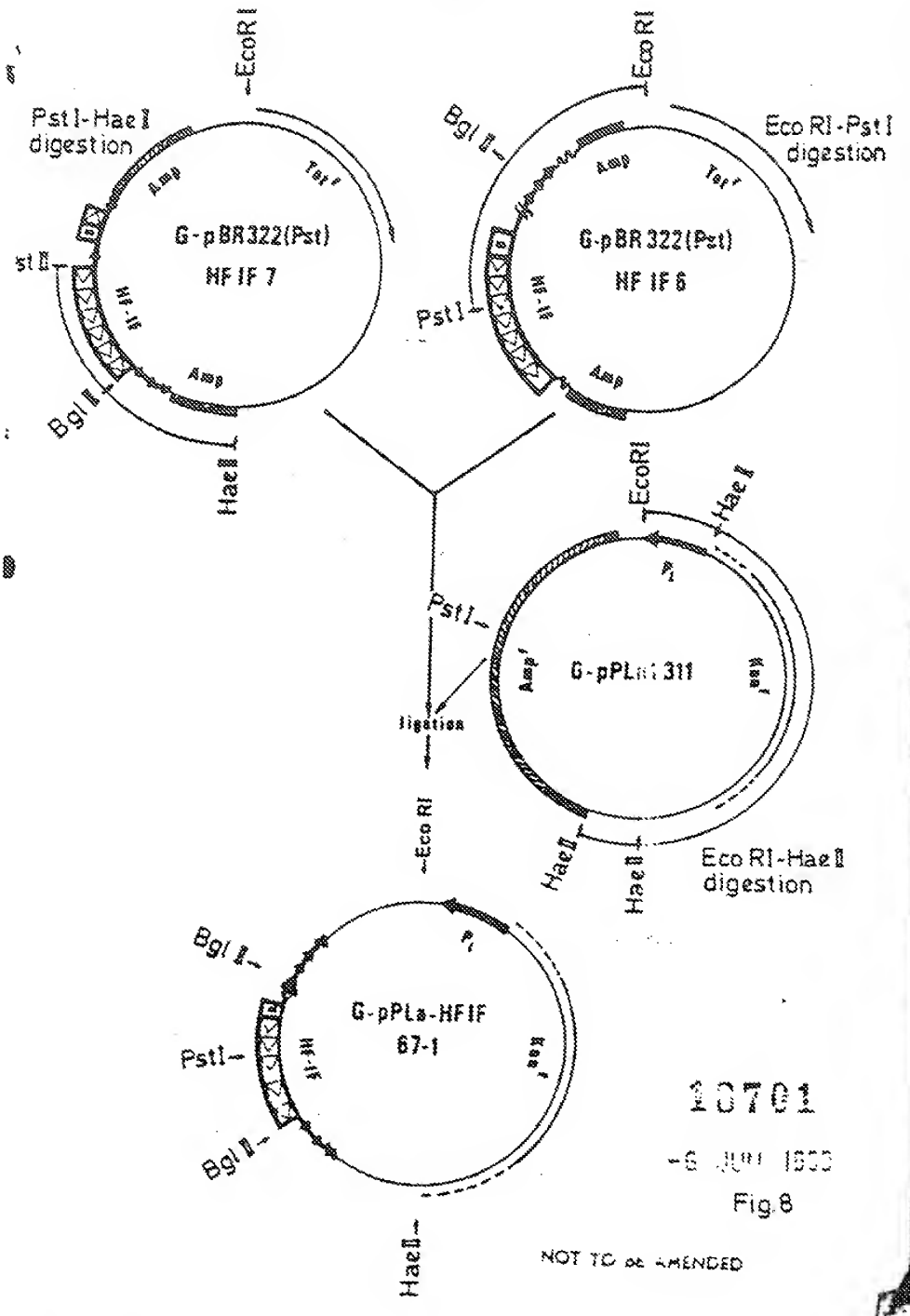


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Fig. 8

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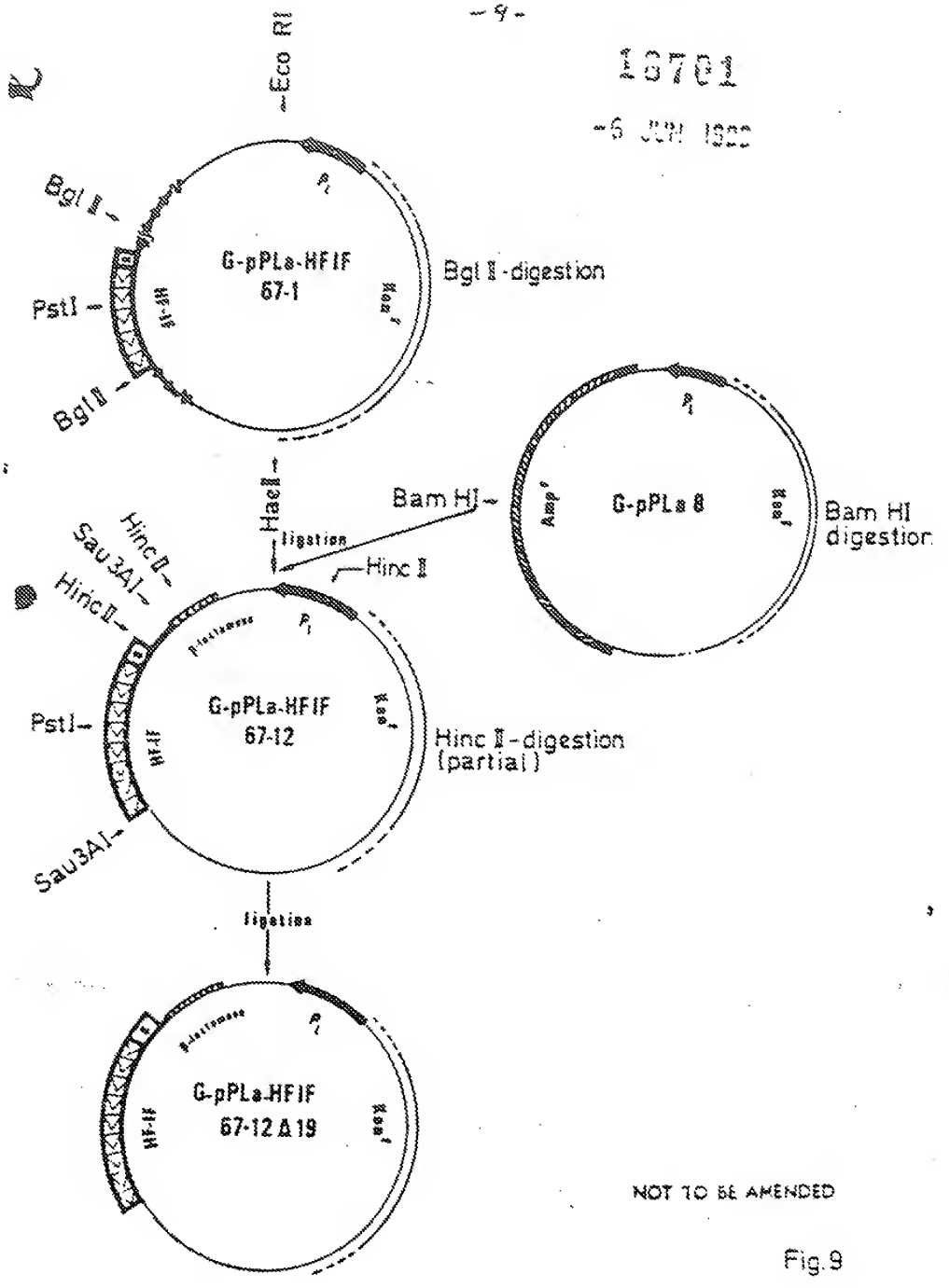

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Fig. 9

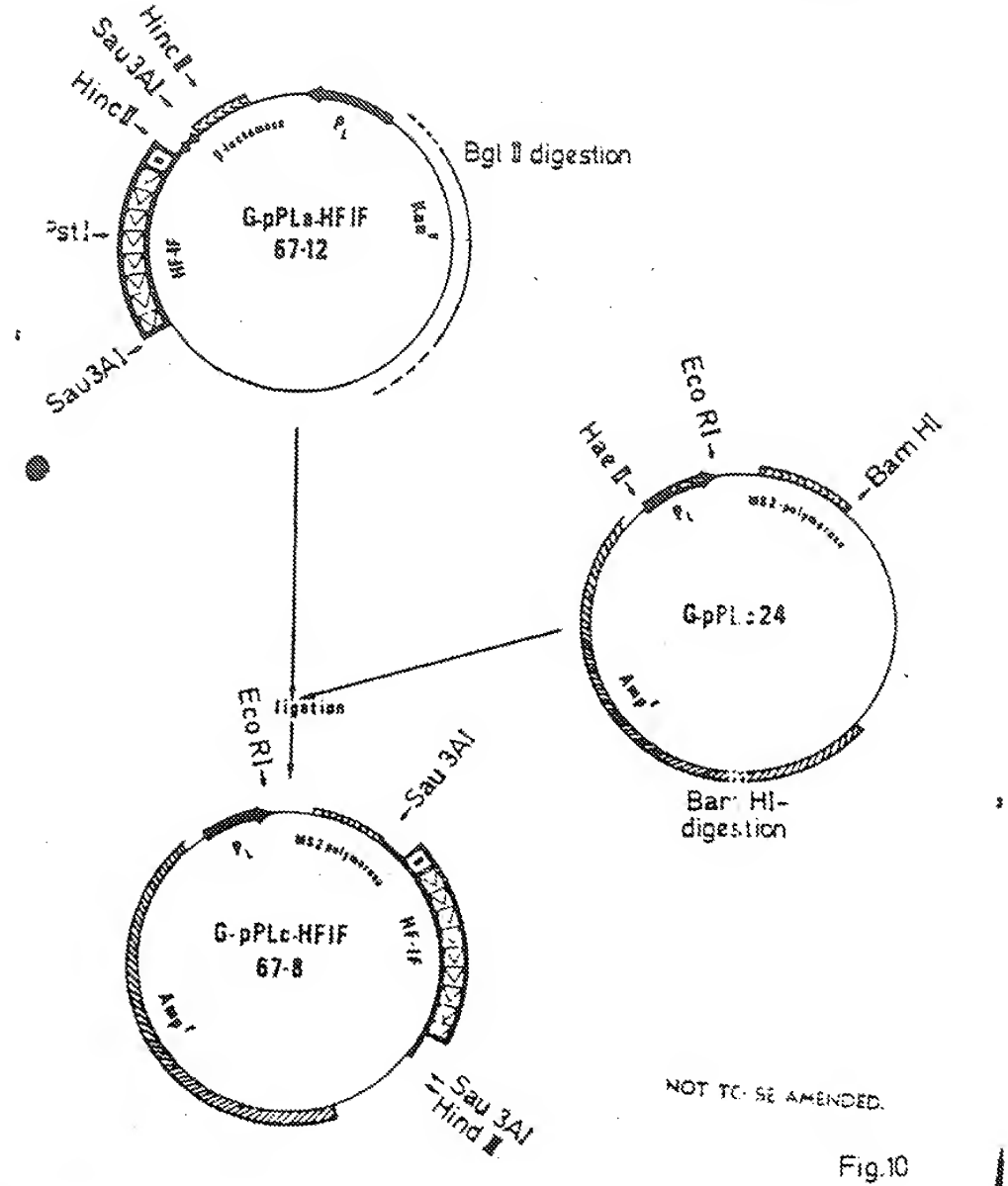

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Fig.10

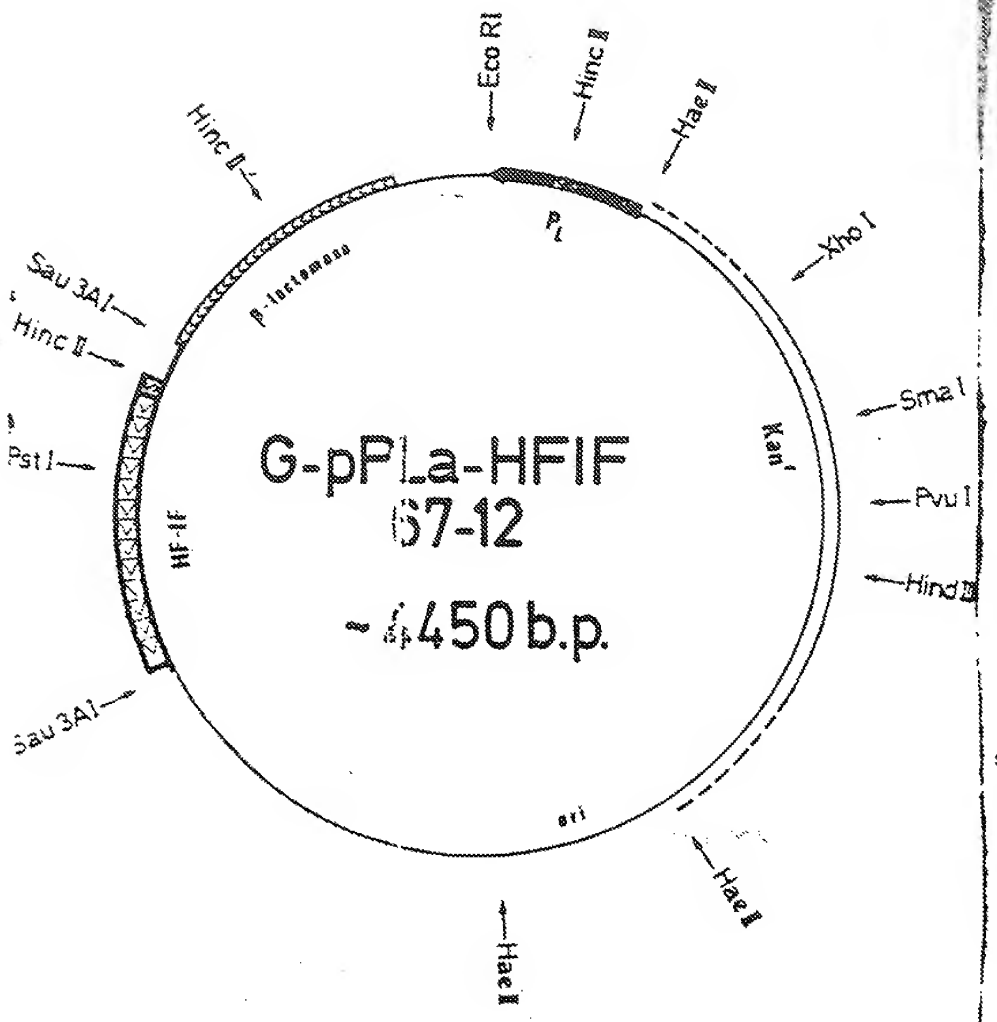

Fig.11

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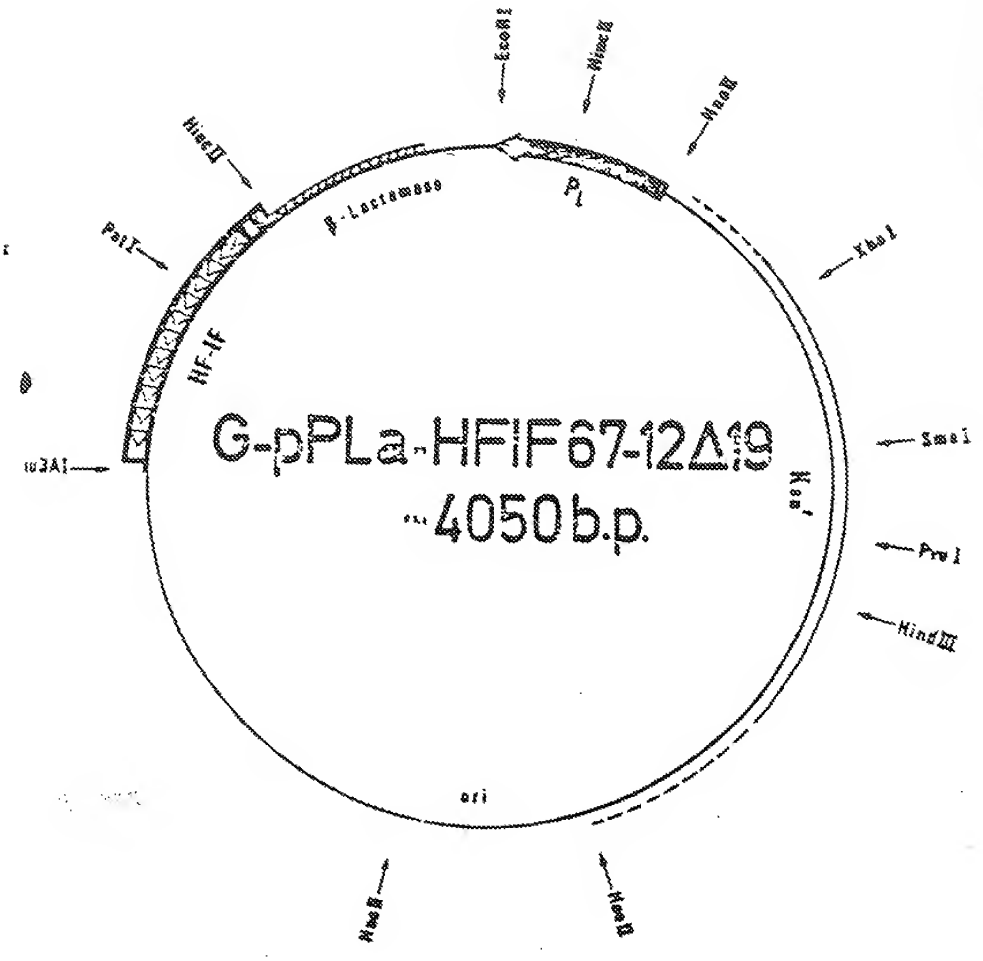

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Fig.12

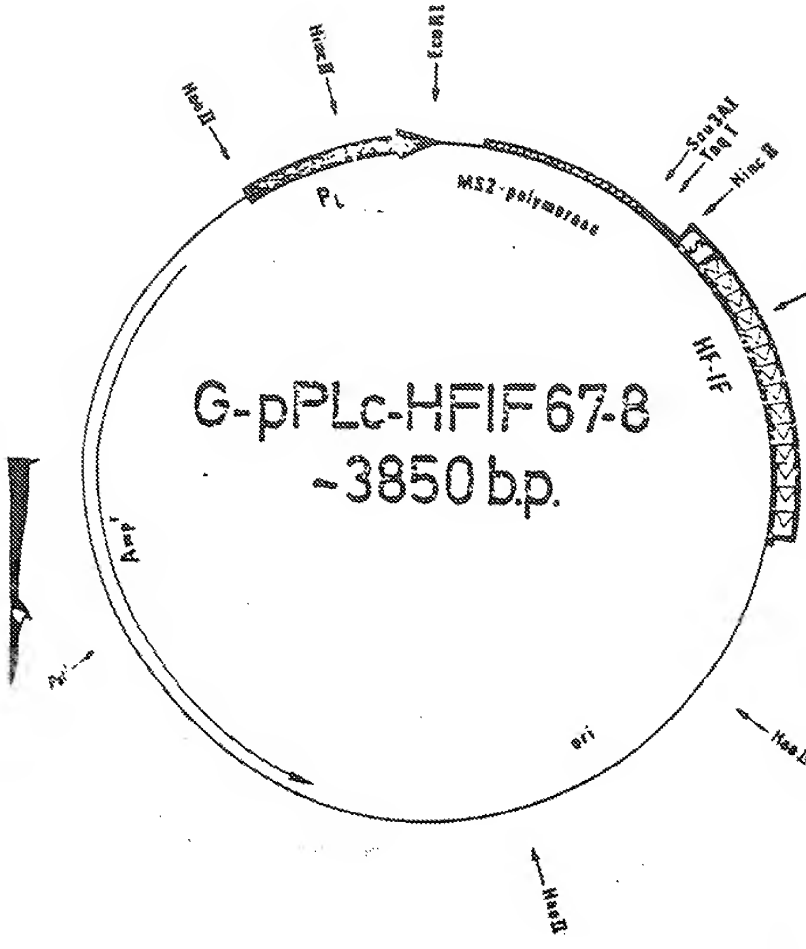

Fig.13